THE SIGNIFICANCE OF FATTY INFILTRATION IN THE DEVELOPMENT OF HEPATIC CIRRHOSIS DUE TO CHOLINE DEFICIENCY

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ONE TEXT FIGURE AND ONE PLATE (FIVE FIGURES)

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It is now well established that ingestion of a diet low in protein and choline will result in hepatic necrosis and cirrhosis in the rat (Gyorgy and Goldblatt, '39, '42; Webster, '41; Blumberg and McCollum, '41; Daft et al., '41). However, before this was demonstrated, Connor ('37) had expressed the view that Laennec's cirrhosis in man is the ultimate outcome of fatty infiltration of the liver and surmised that if a given cirrhotic liver is not fatty, it is only so because the fat previously present has disappeared. Since choline deficiency rapidly results in fatty infiltration of the liver it has seemed reasonable that the hepatic necrosis and cirrhosis of choline deficiency may be the obligatory consequences of chronic fatty infiltration. However, no adequate proof of this hypothesis has been offered. The present paper describes an attempt to determine experimentally the significance of the accumulation of fat in the development of the subsequent hepatic necrosis and fibrosis due to choline deficiency.

EXPERIMENTAL

Weanling rats of the Vanderbilt strain (Wolfe et al., '38) were offered the experimental diets when they had attained

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a weight of 45–50 gm. They were housed in individual cages and, with the exception of group H, were offered the various diets ad libitum. A large number of the animals died before the termination of the experimental period of 120 days. Usually this was recognized in advance and, when it was deemed that such an animal could not survive the night, it was sacrificed by decapitation and the liver excised, in the same manner used for all rats after 120 days. About half the liver was then taken for total fat analysis (Handler and Dann, '42) and the remainder was fixed in neutral 10% formalin. Sections were stained with hematoxylin and eosin.

Optimal dietary conditions for the production of hepatic cirrhosis due to choline deficiency

Before proceeding to examine the role of fatty infiltration it was thought necessary to first establish optimal dietary conditions for the production of hepatic cirrhosis due to choline deficiency. Of the various diets which have been used to this end, the most effective appear to have been those of Daft et al. ('41). These workers employed diets containing 4% casein, with very little fat and starch as the carbohydrate. It was, however, thought desirable to compare such a diet with others (a) using sucrose as the dietary carbohydrate since Griffith ('41) was unable to produce "hemorrhagic kidneys" when starch was the dietary carbohydrate, (b) containing greater amounts of fat and cholesterol, and (c) containing somewhat more protein since this low level permits only very slow growth. The composition of these diets and the others employed in this study are summarized in table 1.

When starch was used as the dietary carbohydrate (group A) on a low fat diet all but nine animals survived the 120-day experimental period. The livers of all this group were found to contain moderate quantities of fat and most of those which were appreciably fatty showed a slight to moderate degree of necrosis. The necrosis appeared to commence around the central vein and was usually restricted to this region. The liver cells showed various stages of necrosis, from hyaliniza-

tion or poor staining of the cell to complete disintegration. The older necrotic areas appeared as foci of loss of liver substance with replacement by masses of refractive non-staining bodies (yellow, yellow-orange, or yellow-green) lying in a minimal amount of scar tissue. This material presumably was identical with the material named "ceroid" by Lillie et al. ('42). It lay either free in scar tissue or within macrophages. The scarring was very slight. The liver cells that remained showed a marked fatty change and were grouped into roughly circular

TABLE 1

Percentage composition of experimental diets.

INGREDIENT	DIET							
	A	B	C	D	E	F	G	L
Casein	5	5	5	7	10	8	5	18
Starch	85.5		68	66	63	63		53.8
Sucrose		85.5						
Lactose							68	
Cottonseed oil	3	3	10	10	10	10	10	10
Lard			10	10	10	10	10	10
Cod liver oil	2	2	2	2	2	2	2	2
Salts[1]	4	4	4	4	4	4	4	4
Cystine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol			0.5	0.5	0.5	0.5	0.5	0.5
Nicotinamide						2		1.2

In addition, to each kilogram of diet were added the following: thiamine chloride 10 mg., riboflavin 20 mg., pyridoxine 10 mg., calcium pantothenate 100 mg., p-aminobenzoic acid 100 mg., 2-methylnapthoquinone 5 mg.

[1] Hubbell, R. G., L. B. Mendel and A. J. Wakeman, 1937 J. Nutrition, vol. 14, p. 273.

islands surrounded by delicate strands of connective tissue consisting of one or two layers of fibroblasts lying next to newly formed capillaries. There was practically no inflammation except for rare tiny foci of round cells and eosinophils. There was no hemorrhage.

The diet of group B differed from that of group A only in that sucrose was the dietary carbohydrate. Of the total of twenty-four animals offered this diet, nineteen died before the end of the 120 period, the mean survival time of this group

being 76 days. Histological study was made of the livers of the survivors and the last ten rats to die. The other animals died unexpectedly and, when found, sufficient post-mortem decay had occurred to warrant discarding them.

Of the livers examined, two showed essentially no fatty change. The others were moderately fatty. However, in only two was there any evidence of necrosis and this was slight. This may have been due to the early death of these animals so that there was not sufficient time for any extensive necrosis or it may be related to the only moderate degree of fatty infiltration. The latter seems reasonable since the rats of group B ate, on the average, 5.1 gm. per day while those in group A

TABLE 2

Summary of histological findings in choline deficiency under various circumstances.

GROUP	MEAN WEIGHT OF SURVIVORS	LIVER WEIGHT	LIVER FATTY ACIDS	HISTOLOGICAL EXAMINATION		
				Fat	Necrosis	Fibrosis
	gm.	*gm.*	*% wet weight*			
A	154	7.9	16.3	++	++	0
B	109	7.6	12.7	+++	+	0
			Highly variable			
C	98	8.1	3–27	++++	++++	++
H	41	2.4	4.4	0–+	0–+	0
K	52	2.0	5.1	0–++	0–+	0
L	154	10.5	23.2	++++	0–+	0

ate 6.6 gm. per day. However, the difference in liver fat concentrations of these two groups, as shown in table 2, was not impressive. The basis for this distinction is not apparent but may lie in a refection-like process facilitated by the presence of starch and providing accessory factors which were not included in the diets. This situation is the reverse of that observed by Griffith ('41) in his studies of renal damage due to choline deficiency. It was concluded that for the purposes of this study starch was preferable to sucrose as the dietary carbohydrate.

Group C was fed a ration containing 10% lard, 10% cottonseed oil and 0.5% cholesterol, with 5% casein and starch making up most of the balance. Of twelve rats in this group,

four survived for the entire experimental period and the mean survival time of the others was 97 days. These animals grew slowly through almost the entire experiment although some lost weight during the last week or two. Four of the animals that died prematurely appeared to gain weight rapidly during the last few days but this was due to the accumulation of ascitic and pleural fluid. The livers of this group showed marked fatty change and extensive necrosis. In some livers there was nothing left but masses of ceroid within scar tissue and there were huge areas in which no liver cells remained. The mass that composed the liver was really the ceroid and other fatty material lying in bands of connective tissue. The latter mass was made up of spindle-shaped fibroblasts in the more recently destroyed areas but in the older areas consisted of pink staining bands resembling collagen. Where liver cells were seen, they were found in small islands surrounded by delicate bands of connective tissue. Most of these remaining liver cells showed considerable fatty change. On the other hand there were a few circular islands of apparently normal liver cells, which appeared to be areas of regeneration. The results of chemical determinations of fat content in this group were quite variable (table 2). Some of the most extensively necrotic livers were found to have only moderate fat concentrations, 3 to 10%, while others were extremely high, 15–25%. When groups of six rats were sacrificed 30 and 60 days after they were put on diet C, liver fat concentrations uniformly above 15% and as high as 30% were found. This must indicate that the necrotic livers of low fat content were at one time highly fatty but the fat disappeared with the death and subsequent phagocytosis of the liver cells. This process, then, is not primarily a fibrosis, but a fat induced necrosis with subsequent scar replacement. It must be recognized that were it not for the ceroid, which is apparently not an integral part of this process but accumulates only when appreciable quantities of cod liver oil are fed (Endicott, '44; Wachstein, '45), these livers would have been much smaller and a larger percentage of their total mass would have been fibrous tissue.

To ascertain the optimal protein content of the diet for the production of hepatic damage due to choline deficiency, groups D and E were fed diets similar to C but in which the casein level was raised to 7 and 10%, respectively. Of the rats in group E, 80% died within the first 12 days of the experiment and in all of these were found the grossly hemorrhagic kidneys associated with acute choline deficiency in the very young growing rat. More surprising, however, was the fact that virtually all of the animals in group D died after 5 to 7 weeks and in these animals also were found grossly hemorrhagic kidneys. Their livers, while very fatty, showed practically no necrosis or fibrosis. This was not surprising in view of the comparatively brief duration of the experiment. These animals will be described in detail in a subsequent report.

Not included in the table were two other groups which received the diet of group C supplemented with 0.35% choline chloride and 0.7% methionine, respectively. One of the ten animals in each group died prematurely but these, like the others, showed normal liver fat concentrations and no evidence of liver necrosis. Under these circumstances, it was concluded that the diet of group C, containing 5% casein, with starch as the carbohydrate and a considerable quantity of fat and cholesterol was optimal for the production of hepatic damage due to choline deficiency.

The effects of choline deficiency without fatty metamorphosis of the liver

The conditions found most favorable for the development of choline deficiency hepatic cirrhosis were also those which most promptly induced the accumulation of excessive liver fat. This is in accord with the principles stated by Connor ('37). To further test this hypothesis it was decided to study the fate of animals on a choline deficient regime so modified that no accumulation of excessive liver fat would occur. Several attempts were made to produce such conditions.

The first of these was excessive nicotinamide feeding. If 2% of nicotinamide is included in an 8% casein, high fat diet

(group F) the animals lose weight and fail to show fatty livers, even though the cause of growth failure is methionine, and consequently choline, deficiency (Handler and Dann, '42; Handler, '44). However, this phenomenon had only been studied in experiments of 14- and 28-day duration. When a longer trial was made, the animals in this group appeared to fall into two classes: those which lost weight steadily and

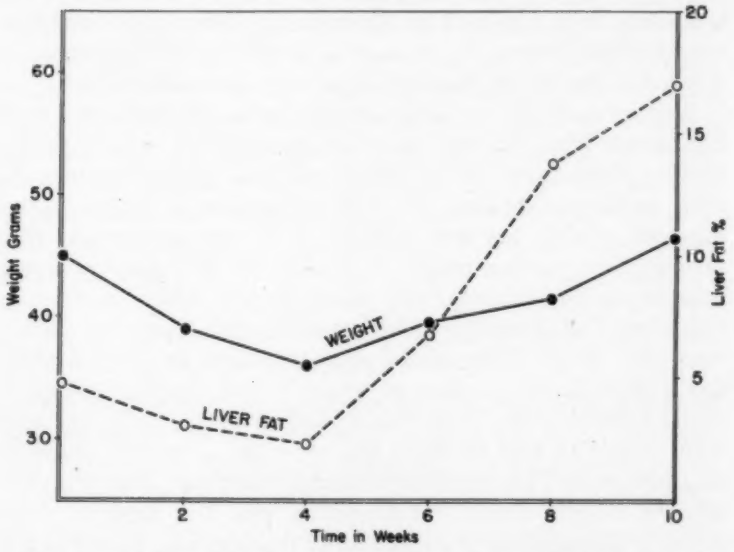

Fig. 1 Relationship between growth and liver fat concentration in excessive nicotinamide feeding.

died, presumably of methionine deficiency, and those which reached and maintained a weight plateau, after which their liver fat concentrations rose steadily. A few animals actually began to gain weight after 4 to 5 weeks and these showed a considerably elevated liver fat content. These data are summarized in figure 1, the figures representing mean values obtained with groups of five rats each sacrificed at 14-day intervals. This procedure, therefore, seemed fruitless for our purposes.

A second attempt was made, utilizing the fact that young rats on a low protein, high fat diet with lactose as the dietary carbohydrate show very little increment in liver fat content and no hemorrhagic kidneys (Artom and Fishman, '45; Handler, unpublished data). However, when a diet similar to that of group C was fed, with lactose substituted for starch, all the animals died after 14 to 22 days, a phenomenon not observed in the earlier experiments of 7-day duration. This has since been reported by Ershoff and Deuel ('44) and the mechanism is now being studied in detail and will be reported later. Consequently, this technique also was discarded.

Rats fed a diet concomitantly deficient in thiamine and choline do not show the characteristic fatty livers of choline deficiency (McHenry, '37). This is not due to any specific effects on fat metabolism, but, rather, appears to be due to the anorexia, and is but one instance of the generalization that rats which are losing weight or in negative nitrogen or caloric balance, do not develop fatty livers despite choline deficiency (Handler, '43; Handler and Bernheim, '43; Boxer and Stetten, '44). Group H consisted of twenty-four rats which were fed a diet similar to that of group C but lacking thiamine. These animals were started at an initial weight of 70 gm. and after they had lost about 20 gm. were given weekly doses of thiamine, which varied from rat to rat, such that in no instance did the weight of any rat exceed 55 gm. and most of them actually dropped below 50 gm. Only thirteen rats survived for 120 days, the others dying, apparently, of thiamine deficiency. The livers of all the rats which died prematurely were found to have unusually low concentrations of liver fat. Of the others, most had small but apparently normal livers. The maximum liver fat concentration found in the group was 7.8%. Histologically these showed little or no fatty infiltration and virtually no necrosis was seen.

Group K was composed of twelve rats which were pair fed with members of group C and fed the same ration, but whose daily food intake was limited to about 50–60% of that of the members of group C. Two of these failed to survive the ex-

perimental period. Of the remainder, four actually gained some weight over this period, albeit very slowly, and the remainder either lost weight or remained almost constant. The animals which did gain in weight were found to have somewhat elevated liver fat concentrations while the others were normal or almost so. No necrosis or fibrosis was observed in the livers of normal fat concentration, while only a slight degree of focal necrosis with minimal fibrosis was found in the livers of elevated fat content. Groups H and K, then, afford examples of situations in which rats were maintained for lengthy periods on choline deficient regimes but under circumstances in which virtually no accumulation of excessive fat occurred, nor was there any appreciable necrosis or fibrosis. This suggests that hepatic necrosis, in choline deficiency, not only occurs under those circumstances which produce an extensive fatty metamorphosis but may actually be the end result of this process.

The effects of choline deficiency on rats fed a relatively high casein diet

All of the diets used above, while choline deficient, were complicated by the fact that they were, of necessity, low protein diets. This was done to keep the methionine content at a minimum. Choline deficiency on relatively high protein intake has only been studied with fibrin or peanut meal rather than casein as the dietary protein. It was thought of interest to study the effects of choline deficiency in the presence of an adequate level of casein intake. Group L, therefore, was offered an 18% casein diet whose effective methionine content was reduced by the addition of nicotinamide. Thirty-six rats were used and batches of six each were killed at the end of 2, 4, 8, and 12 weeks and their livers taken for fat analysis. Two animals died unexpectedly and their livers were included with those of the remaining ten which were sacrificed after 120 days and samples taken for both chemical and histological examination. As shown in table 3, this device was successful

in that high liver fat concentrations were maintained throughout the experimental period. An attempt was also made to determine the effective methionine content of this diet. This was done by placing groups of two rats each in metabolism cages and urine collections were made for a 48-hour period and the N′-methylnicotinamide content estimated. While the first values were determined by the procedure of Huff and Perlzweig ('43), the later figures were obtained by the method of Huff, Perlzweig and Tilden ('45). Recovery experiments after giving known amounts of N′-methylnicotinamide chloride to normal rats of comparable size were also performed. When

TABLE 3

Liver fat concentration during course of moderate nicotinamide feeding on a high casein diet.

TIME	RAT WEIGHT	LIVER FAT	TIME	RAT WEIGHT	LIVER FAT
days	*gm.*	*% wet weight*	*days*	*gm.*	*% wet weight*
0	43	4.9	56	112	22.3
14	64	12.0	84	137	22.4
28	92	18.1	120	154	23.2

nicotinamide is given to rats, about one-fifth of the dose can be recovered in the urine as N′-methylnicotinamide (Huff, '45). Furthermore, when the latter compound itself is given, only about one-fifth of it can be recovered in the urine. From these considerations, it has been surmised that the four-fifths of unrecovered nicotinamide was also methylated and this compound then destroyed or further altered. Therefore, in table 4 are presented two columns representing the amount of available methionine if this assumption is correct (B) and also based directly on the urinary content of N′-methylnicotinamide (A). The methionine content of casein was assumed to be 3.5% (Block and Bolling, '45). The urinary recovery of N′-methylnicotinamide, as a percentage of the administered nicotinamide, decreased somewhat with time. It cannot be stated whether this represents decreased synthesis or increased destruction of the N′-methylnicotinamide. Through

most of the experiment, however, the effective methionine content of the diet was equivalent to a diet containing either 15% or 6% casein, depending on the validity of the assumption above. The extremely fatty livers make the latter values seem more nearly correct.

TABLE 4

The amount of effective methionine in a diet containing excess nicotinamide. All values are expressed in terms of one rat for 1 day.

TIME	FOOD INTAKE	METHIONINE IN FOOD	NICOTINAMIDE INTAKE	METHYLNICOTINAMIDE EXCRETED	EFFECTIVE METHIONINE		METHYLNICOTINAMIDE INTAKE
					A	B	
day	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
28	8.3	58	100	7.8	50	16	...
118	11.5	73	138	8.6	64	26	...
1	...	..	...	38	..	..	220

TABLE 5

Unavailability of N′-methylnicotinamide for choline and methionine synthesis. All values represent means for groups of 6 rats, for an experimental period of 7 days.

SUPPLEMENT	WEIGHT GAIN	FOOD INTAKE	LIVER FATTY ACIDS
	gm. per day	*gm. per day*	*% wet weight*
None	2.8	7.8	21.6
Choline	2.8	7.7	5.1
Methionine	3.1	8.2	4.6
N′-methylnicotinamide	2.5	6.9	18.0
N′-methylnicotinamide + homocystine	2.3	7.2	18.7

The calculations and assumptions above predicated that the methyl group of N′-methylnicotinamide was not available for the resynthesis of methionine or choline. This has been indicated by the work of Handler and Dann ('42) but more concrete evidence is presented here. Group 1 was fed diet E, similar to that of group C but containing 10% of casein. Group 2 received the same diet with a supplement of 0.5% choline; group 3 received 0.7% methionine; group 4 received 1% of N′-methylnicotinamide chloride, while group 5 received 1% N′-methylnicotinamide plus 0.7% dl-homocystine. The data are summarized in table 5. It will be seen that while choline

and methionine completely prevented the excess liver fat accumulation, N′-methylnicotinamide alone or in combination with homocystine failed to exert lipotropic activity.

Of the twelve rats in group L sacrificed at the end of 120 days, five showed only minimally fatty livers with no necrosis or fibrosis. The remaining seven livers were extremely fatty yet showed only very slight necrosis with minimal fibrosis. The necrosis, such as it was, appeared to commence around the central vein. The changes were manifest as hyalinization and poor staining of the liver cells with subsequent loss of liver parenchyma and accumulation of ceroid within a slight amount of scar tissue. This group then, stands in contrast to groups A and C. Despite the persistent fatty degeneration only a minimal amount of necrosis was evident when the animals were sacrificed. It seems likely that the necrosis might have been more extensive if the experiment had been prolonged, but the results would not then have been comparable with groups A and C. Thus, while the accumulation of fat in the livers of animals on low protein diets, in analogy with human alcoholic or Laennec cirrhosis, appears to lead to necrosis with subsequent fibrosis, a reasonably high protein intake seems to afford a degree of protection against the deleterious effects of fat accumulation noted on low protein diets.

DISCUSSION

In agreement with all previous reports it was found that high fat, low protein diets provide optimal conditions for the production of hepatic necrosis and fibrosis. These conditions also proved optimal for the accumulation of liver fat. When these conditions were altered either by thiamine deprivation or simply restricting food intake the fatty infiltration was minimal and virtually no necrosis was observed. This suggests that the liver necrosis of choline deficiency may be the obligatory consequence of massive fatty infiltration of the parenchymatous liver cells and that only the lipotropic activity of choline is involved in maintaining the integrity of the liver.

This is in accord with Connor's ('37) concept of the pathogenesis of alcoholic cirrhosis in man.

The data reported herein do not afford an explanation of the mechanism by which the accumulation of fat may result in the death of parenchymatous liver cells. It is certain that such cells are highly organized systems and that the integrity of this organization is necessary for the proper functioning of the cell. The fat within liver cells is either absorbed from the surrounding medium or is synthesized de novo from carbohydrate and protein precursors. However, to leave the liver as phospholipid, choline is necessary and, in choline deficiency this fat accumulates, appearing first as small discreet droplets which later coalesce. As more fat collects the cytoplasm and nucleus can be found together in one corner of the cell whose bulk is now largely the fat droplet. However, as still more fat appears, it seems not unreasonable to believe that the physical organization of the cytoplasm and nucleus is disrupted with consequent loss of function and death. With the disintegration of the cell the fat is removed by phagocytosis and scar tissue then develops in its place.

On the other hand the behavior of the rats of the high casein, nicotinamide containing diets has demonstrated that liver cells may survive despite prolonged fatty infiltration. These data are in contrast with those obtained after feeding diets containing relatively large amounts of fibrin or peanut meal. The fat concentrations in the livers of group L were as great as those seen during the development of liver necrosis and fibrosis under our optimal conditions. Yet virtually no necrosis was found. No evidence is available concerning the fate of the methionine moiety after transmethylation and N′-methylnicotinamide synthesis although it has been assumed that homocysteine is formed and the latter converted to cysteine and cystine. It remains possible that the apparent protection afforded the liver by this high casein diet resides in this methionine derivative, but this protective capacity is not based upon lipotropic activity. It is, however, consistent with Gyorgy's ('44) findings that choline plus cystine is more

effective than choline alone in the prevention of necrosis and cirrhosis, and perhaps this situation is similar to the frequent post-mortem finding of highly fatty livers with little or no necrosis. Further study of the possible role of other amino acids and such factors as strepogenin is warranted.

Recently Himsworth and Glynn ('44 a, b) have distinguished between an acute, massive, hepatic necrosis, analogous to acute yellow atrophy in man, which they state to be due, specifically, to methionine deficiency (but despite rather than because of its lipotropic activity) and a progressive, diffuse hepatic fibrosis which is not accompanied by necrosis but thought to be due to fatty infiltration caused by lack of sufficient dietary lipotropic factors (methionine, choline, etc.) and preventable by dried yeast. Comparison of their data with ours is difficult because of the many differences in technique and the diets employed. Since the lesions we have studied were actually post-necrotic scars they would consider our results to be the combination of their two supposedly, independent, disease processes. It is difficult indeed to understand the development of scar tissue in a liver which is composed of living, albeit fatty cells. We have observed no process similar to their massive necrosis and believe that this is because of the inclusion of sufficient cystine in all of our diets. While they found that methionine was preventive but cystine was not, it must be noted that while they used supplements of 20 mg. per day of methionine, they tried cystine only at the level of 2.2 mg. per day. In contrast our diets provided about 30 mg. of cystine per day which should have been ample to prevent the specific necrosis of cystine deficiency (Weichselbaum, '35; Daft et al., '41) which is apparently similar to these authors' acute, massive necrosis. The protective action of methionine may well have been due to its conversion to cystine. Since the livers of our choline-deficient rats were definitely necrotic despite the presence of adequate dietary cystine, and since choline supplements to such a diet completely prevented the appearance of any form of demonstrable hepatic damage, we must conclude that the hepatic fibrosis of choline deficiency is a form of postnecrotic scarring.

SUMMARY

1. Diets high in fat containing 4–5% casein with starch as the carbohydrate and supplemented with cystine and cholesterol proved most suitable for the production of choline deficiency liver necrosis and fibrosis in albino rats.

2. Thiamine deficiency or restricted food consumption prevented the accumulation of excessive liver fat and also liver necrosis.

3. N′-methylnicotinamide was found to exert no lipotropic activity alone or in combination with homocystine.

4. Inclusion of excess nicotinamide in an 18% casein diet produced extensive fatty infiltration but only minimal necrosis of the liver.

5. It is suggested that the hepatic necrosis and fibrosis of choline deficiency may be the result of chronic fatty infiltration. The ingestion of an adequate quantity of good protein protects the liver from the deleterious effects of chronic fatty infiltration but this protective capacity is not based upon lipotropic activity alone.

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PLATE 1

EXPLANATION OF FIGURES

All sections were stained with hematoxylin and eosin.

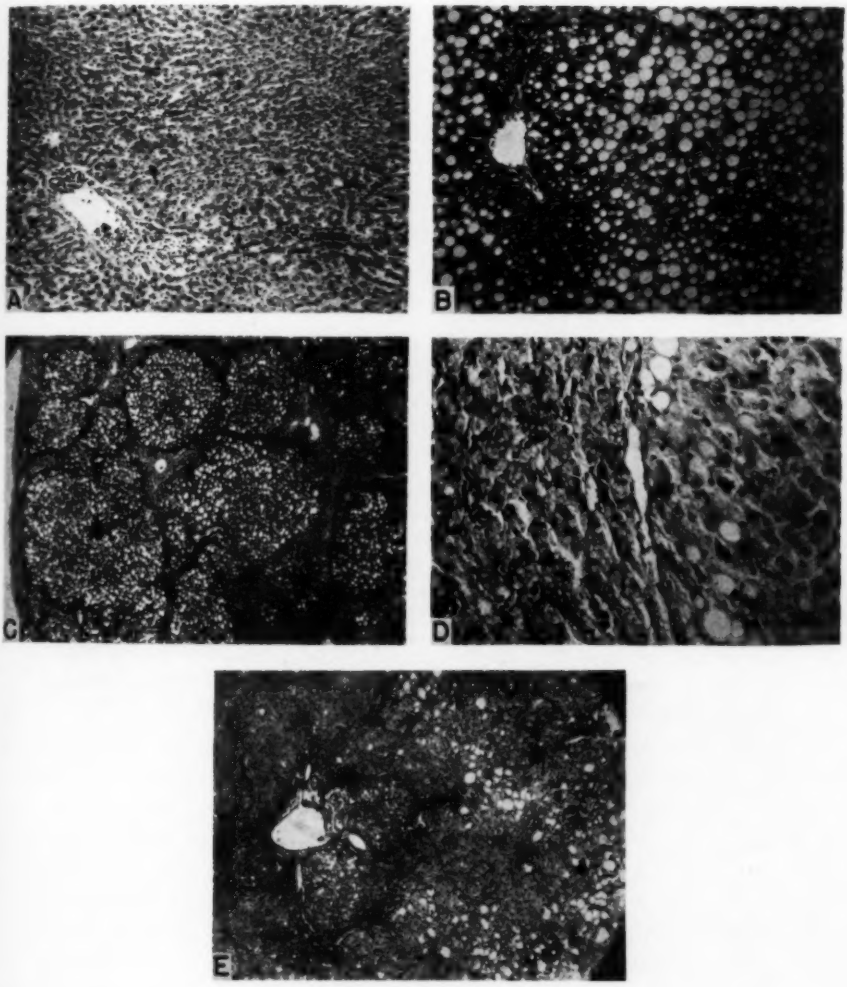
A. Normal liver; group K; restricted intake of optimal cirrhosis producing diet. × 137.

B. Liver of typical rat in group L; nicotinamide added to high casein diet; note lack of necrosis despite extensive fatty change, especially in central portion of lobule. × 137.

C. Liver of rat in group C; optimal diet for producing cirrhosis. This liver still shows a marked fatty change with but a moderate degree of cirrhosis. The liver substance is arranged in irregular islands demarcated by connective tissue in which the ceroid is visible as non-staining refractile white dots. × 60.

D. Another liver from group C. This area is at the periphery of an island of liver tissue and shows the numerous globules of ceroid lying in a meshwork of newly formed connective tissue and capillaries. × 295.

E. Another liver from group C. This is an extreme degree of liver necrosis. In the area shown practically all the liver cells have died so that the mass consists of small globules of ceroid lying in connective tissue. Some globules of fat still remain. × 60.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VITAMIN C CONTENT OF MARKET MILK, EVAPORATED MILK, AND POWDERED WHOLE MILK

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TWO FIGURES

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The vitamin C content of cows' milk has been studied by a large number of investigators. Booher, Hartzler and Hewston ('42) and Mendive and Repetto ('40) have made compilations of values found in the literature. Much of the data concerning the vitamin C content of milk has been obtained with the object of studying factors which influence the amount originally present in the milk and its stability under various conditions. Comparatively little data have been published concerning the actual amount of vitamin C in milk and milk products produced commercially and sold to consumers.

An extensive survey of the ascorbic acid content of commercial pasteurized bottled milk was conducted by Guthrie, Hand, and Sharp ('38), who found an average of 2.9 mg. reduced ascorbic acid per liter as the result of analyzing 1502 samples from dairy plants in the State of New York 24 hours after delivery. Hawley ('38) found an average of 9 mg. reduced ascorbic acid per liter in 100 samples of commercial pasteurized milk taken from milk wagons in Rochester, New York. Samples from five dairies in Madison, Wisconsin, were analyzed by Woessner, Elvehjem and Schuette ('39) and found to contain an average of 8.9 mg. reduced ascorbic acid and 12.6 mg. total vitamin C per liter. Among those studies

made on milk sold in foreign cities is that of Mendive and Repetto ('40), who found 11.3 mg. to 22 mg. total vitamin C per liter in pasteurized milk sold in Buenos Aires, approximately half being in the form of dehydroascorbic acid; Palladina and Anashkina ('37) found an average of 16.5 mg. vitamin C per liter in milk sold in Leningrad.

The importance of dehydroascorbic acid as a source of vitamin C has not been realized, nor suitable methods for its analysis devised until recent years. The necessity for determining dehydroascorbic acid in addition to reduced ascorbic acid has been shown by Hand ('43), who states that determinations of reduced ascorbic acid alone may be in error as a measure of total vitamin C by amounts ranging from 0 to 6 mg. per liter.

Sharp, Guthrie and Hand ('38, '40, '41, '42) have made comprehensive studies of the stability of vitamin C in milk. A total of 1187 samples of fresh milk were analyzed in which the reduced ascorbic acid content was found to range from 7 to 41 mg. per liter with an average value of 22.2 mg. Pasteurization was found to accelerate the destruction of ascorbic acid only in the presence of dissolved oxygen and copper. Season and feed of the cow exerted little effect upon the ascorbic acid content of the fresh milk. The same authors studied losses of vitamin C in milk in commercial milk plants from the time of its delivery to the country milk plant to bottling in New York City. Milk in the patrons' cans at the country shipping plant averaged 18.9 mg, per liter, weigh tanks 18.5 mg., pump reservoir 18.4 mg., tank truck 18.3 mg., and tank cars 17.7 mg. In one plant in New York City, using holder pasteurization, the milk entering the weigh tank averaged 15.7 mg., before pasteurizer 15.7 mg., after pasteurizer 11.1 mg., after cooler 9.1 mg. and in the bottle 9.1 mg. A similar study as the milk passed through a New York City plant using short-hold-high-temperature pasteurization showed that milk entering the plant averaged 15.7 mg., after pasteurization 15.0 mg., and in the bottle 14.3 mg. The causes for losses in vitamin C are shown to be the result of copper contamination

and presence of dissolved oxygen, during pasteurization and especially on holding after pasteurization.

The vitamin C content of evaporated milk has been stated by various investigators (Doan and Josephson, '43; Henry et al., '38; Meulemans and de Haas, '38; Schlemmer et al., '32; Taniguchi et al., '37; Tomoi and Tomita, '37; and Woessner et al., '40) to range from practically none to more than that found in fresh milk. It has been pointed out by Woessner, Elvehjem and Schuette ('40) that in most of the earlier work methods were employed in which there was no evidence presented as to their specificity for vitamin C. This no doubt explains for the most part the wide variation in values found in the literature. More recent values obtained with methods designed to correct for interfering substances are those of Woessner, Elvehjem and Schuette ('40) who found 1.9 mg. to 11.2 mg. per liter of reconstituted evaporated milk purchased from stores, while an average of 4.1 mg. per liter was obtained by Doan and Josephson ('43).

Although there are a number of references in the literature (Barcroft, '43; Henry et al., '39; Hochberg et al., '43; Jung, '40; Meulemans and de Haas, '38; Renner, '36; Schlemmer et al., '32; Tomoi and Tomita, '37; and Woessner et al., '40), almost no comprehensive data are available concerning the vitamin C content of powdered whole milk. Analysis has generally been carried out on only a few samples and values vary greatly among different investigators. The same criticism as to lack of evidence for the specificity of the methods employed for the analysis of evaporated milk can be applied to most of the data for vitamin C in powdered milk.

METHODS

Reduced ascorbic acid in market milk was analyzed by indophenol titration according to the method of Sharp ('38). Dehydroascorbic in market milk was determined after conversion to reduced ascorbic acid with a suspension of Escherichia coli as described by Gunsalus and Hand ('41).

Evaporated milk contains large amounts of reducing substances which interfere with the indophenol titration method. This is also true to a lesser degree with powdered whole milk. To obtain true values for vitamin C the authors' selective oxidation-reduction method (Stewart and Sharp, '45) was used. In this method the enzyme (or enzymes) in cucumber juice is used to destroy interfering substances, while also converting reduced ascorbic acid to dehydroascorbic acid. By subsequently adding a suspension of Escherichia coli or Staphylococcus albus the ascorbic acid is recovered in the reduced form and any dehydroascorbic acid originally present is also converted to reduced ascorbic acid, which can be titrated with indophenol dye.

EXPERIMENTAL AND RESULTS

Market milk

Studies were made to determine the total vitamin C content of milk under conditions representative of those in the consumer's home. For this purpose, samples of commercial pasteurized milk were brought to the laboratory for analysis directly from homes of actual subscribers to home delivery milk and from retail stores. Since much of the milk was delivered during the afternoons, the samples were placed in a refrigerator at 10°C. and analyzed the next morning following date of delivery. This time was taken as the most probable for use in the home. A total of 364 samples were analyzed — 237 quart cartons sold in retail stores and 127 quart glass bottles sold on home delivery routes.

This survey was made in the San Francisco — Oakland — Berkeley Bay Region from May 20 to September 21, 1943. Ten major delivery plants were represented, supplying approximately 80% of the milk sold in this region.

Table 1 gives the average values for each of the 10 plants. The variation in average total vitamin C among the plants was 2.9 mg. to 10.0 mg. per liter. Most of this variation was in the reduced ascorbic acid content, which ranged from 0.8 mg. to

7.9 mg. per liter, dehydroascorbic acid varying only from 1.7 mg. to 3.5 mg. per liter.

The reduced ascorbic acid content of all 364 samples averaged 3.4 mg. per liter, dehydroascorbic acid 2.4 mg. per liter, and the sum of the two, or total vitamin C, 5.8 mg. per liter. Individual samples ranged from 0 to 14.3 mg. reduced ascorbic acid, 0 to 8.2 mg. dehydroascorbic acid and 0 to 15.2 mg. total vitamin C per liter.

TABLE 1

Vitamin C content of commercial pasteurized milk consumer samples of day following date of delivery.

PLANT NO.	NO. OF SAMPLES	TYPE OF CONTAINER (1 QUART)	PLANT AVERAGE VALUES		
			Reduced ascorbic acid	Dehydro-ascorbic acid	Total vitamin C
			mg./liter	*mg./liter*	*mg./liter*
1	29	carton	7.9	2.1	10.0
2	26	carton	4.2	2.1	6.3
3	24	carton	6.4	1.9	8.3
4	27	carton	4.8	2.8	7.6
5	24	carton	4.1	3.3	7.4
6	27	carton	1.2	2.7	3.9
7	23	carton	2.1	1.7	3.8
8	28	carton	1.0	2.7	3.7
9	29	carton	1.5	2.2	3.7
10	36	bottle	1.0	1.9	2.9
1	40	bottle	5.0	2.6	7.6
2	29	bottle	3.8	2.3	6.1
8	22	bottle	0.8	3.5	4.3
Total	364	Ave.	3.4	2.4	5.8

There is a very marked tendency for samples of milk with low reduced ascorbic acid content to contain relatively large amounts of dehydroascorbic acid. Also samples low in dehydroascorbic acid tend to be high in reduced ascorbic acid. This is shown in figure 1, where the largest proportion of samples fall in the lower ranges relative to reduced ascorbic acid and dehydroascorbic acid, but total vitamin C, the sum of reduced ascorbic acid and dehydroascorbic acid, maintains

a fairly constant distribution up to and including the 8 to 10 mg. interval.

In order to further simulate conditions existing in the homes of milk consumers, a total of 110 samples — 44 bottles and 66 cartons — were held another day in a household type of electric refrigerator at approximately 10°C. and again analyzed for vitamin C. A loss of 30% total vitamin C resulted from this additional day's holding.

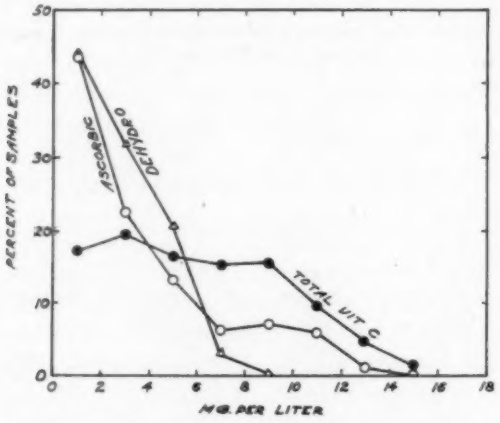

Fig. 1 Vitamin C distribution curves for 364 samples of market milk from consumers' homes and retail stores.

Evaporated milk

Samples of evaporated milk were purchased in retail stores located in San Francisco and Oakland during the months of April, 1943, January, 1944 and May, 1945. Twelve major brands were represented in a total of twenty-five samples analyzed.

Table 2 shows that evaporated milk contains only small amounts of reduced ascorbic acid and little or no dehydroascorbic acid. Reduced ascorbic acid averaged 1.9 mg., dehydroascorbic acid 0.1 mg., and total vitamin C 2.0 mg. per liter of reconstituted evaporated milk. For individual samples the range in

TABLE 2

Vitamin C content of commercial evaporated milk, reconstituted by diluting with equal part of water.

BRAND	NO. OF SAMPLES	ASCORBIC ACID	DEHYDRO-ASCORBIC ACID	TOTAL VITAMIN C
			mg./liter	
1	3	2.2	0.0	2.2
2	1	0.3	0.0	0.3
3	3	2.9	0.6	3.5
4	3	0.7	0.3	1.0
5	2	0.8	0.0	0.8
6	3	2.6	0.2	2.8
7	2	0.4	0.0	0.4
8	1	4.3	0.0	4.3
9	3	0.5	0.2	0.7
10	1	3.8	0.0	3.8
11	2	0.5	0.1	0.6
12	1	3.7	0.3	4.0
Total	25	Ave. 1.9	0.1	2.0

reduced ascorbic acid was 0.0 mg. to 6.6 mg., dehydroascorbic acid 0.0 to 1.3 mg. and total vitamin C 0.0 to 6.9 mg. per liter.

Powdered whole milk

A total of 2890 samples of spray process powdered whole milk were analyzed for total vitamin C. The samples were not over a few days old and were obtained chiefly from three plants, although over 100 samples were obtained from a number of other plants over the country. Also a total of 323 samples were analyzed after storage at room temperature in one pound, air packed tins for 3 months, 651 samples after 6 months' storage, and 400 samples after 12 months' storage. Each sample represents powdered whole milk dried from a separate batch of milk.

The vitamin C in powdered whole milk is largely in the form of reduced ascorbic acid, little or no dehydroascorbic acid being present. For this reason, and for convenience, the data have been expressed in terms of total vitamin C without reference to relative proportions of the two forms. Values are

given in terms of milligrams total vitamin C per 125 gm. powdered whole milk, or the amount required to make one liter of reconstituted whole milk.

The mean average of the 2890 samples of fresh powdered whole milk for the period December, 1942, through May, 1945, was 12.5 mg. vitamin C per 125 gm. The value of 12.3 mg. per 125 gm. was obtained by averaging the data by months. This is shown by the data given in table 3.

TABLE 3

Vitamin C content of fresh spray process powdered whole milk.

MONTHS	NO. OF SAMPLES	MEAN AVE. MG./125 GM.
Dec., Jan., Feb., 1942–1943	128	11.8
Mar., Apr., May, 1943	96	10.9
June, July, Aug., 1943	237	11.4
Sept., Oct., Nov., 1943	107	12.9
Dec., Jan., Feb., 1943–1944	247	12.0
Mar., Apr., May, 1944	211	12.3
June, July, Aug., 1944	144	13.2
Sept., Oct., Nov., 1944	110	13.2
Dec., Jan., Feb., 1944–1945	466	12.8
Mar., Apr., May, 1945	1144	12.8
Total	2890	Ave. 12.3

The combined data are presented in the form of a distribution curve by figure 2. The median value was 12.6 mg. vitamin C per 125 gm. powdered whole milk. Only 4 samples contained less than 5 mg. whereas 2726 (94.3%) contained over 10 mg. per 125 gm.

To study the effect of storage on the vitamin C content of powdered whole milk, samples were analyzed fresh and were air packed in either duplicate or triplicate in one pound tin cans and stored at room temperature (22°C.) for periods up to 12 months. Although in every case the same sample was analyzed fresh and also at the end of a certain storage period, in many instances the same sample was not analyzed at each of the three storage periods. The data are presented in table 4.

After storage for 3 months, the average loss in vitamin C was only 1.4 mg., after 6 months, the loss was 1.8 mg., and after 12 months the loss was 2.5 mg. Even smaller losses in vitamin C have been found in the case of samples which were nitrogen packed.

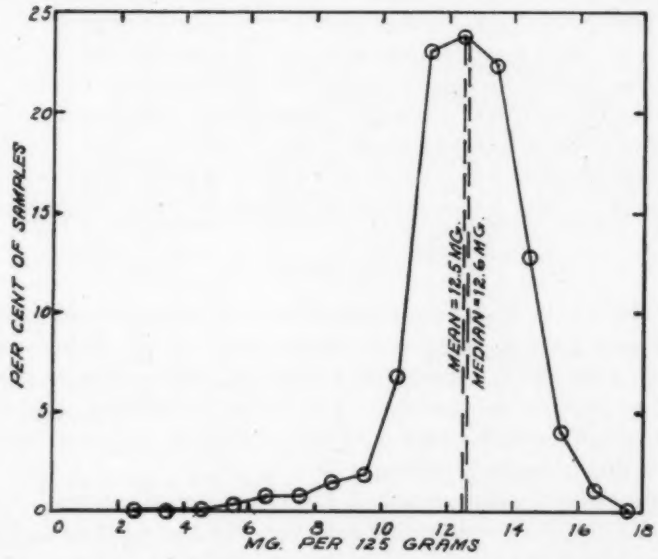

Fig. 2 Vitamin C distribution curve for 2890 samples of fresh spray-process powdered whole milk.

TABLE 4

Effect of storage at room temperature (22°C.), air packed, on the vitamin C content of spray process powdered whole milk.

PERIOD OF MANUFACTURE	NO. OF SAMPLES	VITAMIN C — MG./125 GM.			
		Fresh	3 Mos.	6 Mos.	12 Mos.
Dec., 1942–Feb., 1944	323	12.4	11.0		
Dec., 1942–Sept., 1944	651	12.4		10.6	
Dec., 1942–March, 1944	400	12.3			9.8

Raw milk received at plants

In order to compare values for raw milk with those obtained for market milk, evaporated milk and powdered whole milk, reduced ascorbic acid was determined in a number of samples taken from milk delivered to milk plants by various patrons. Dehydroascorbic acid was not determined, since it is so unstable to pasteurization that it cannot be preserved under even the most ideal processing conditions. A total of 1050 samples were analyzed during five separate periods upon receipt at various plants. The average reduced ascorbic acid content for the five periods ranged from 16.1 mg. to 18.6 mg. with an overall average of 17.1 mg. per liter. This is slightly less than the value of 18.9 mg. found previously by Sharp, Guthrie and Hand ('40).

DISCUSSION

Market milk from consumers' homes and from retail stores averaged 3.4 mg. reduced ascorbic acid, 2.4 mg. dehydroascorbic acid and 5.8 mg. total vitamin C per liter with 30% loss on holding another day. The value for reduced ascorbic acid is in good agreement with that of 2.9 mg. per liter found by Guthrie, Hand and Sharp ('38).

Evaporated milk contained 1.9 mg. reduced ascorbic acid and 0.1 mg. dehydroascorbic acid per liter reconstituted. The value found for vitamin C in evaporated milk is lower than most of the results found in the literature. Our experience with the methods previously used has shown them to give slightly high results in some instances since interfering substances were not entirely eliminated.

The mean value for fresh spray process powdered whole milk was 12.5 total vitamin C per liter reconstituted (125 gm. powder). The median value for the same samples was 12.6 mg. per liter. No extensive data could be found in the literature with which to compare these values. Woessner, Elvehjem, and Schuette ('40) found 10.6 mg. total vitamin C in one sample of spray powdered whole milk, of which 9.4 mg. was in the form of reduced acid. Henry, Houston, Kon, and Os-

borne ('39) found 15.5 mg. total vitamin C per liter reconstituted, which is probably too high since it includes 4.6 mg. dehydroascorbic acid obtained by a method tending to give high results. Other values reported in the literature, mostly on a few isolated samples are 16.9 mg. (Tomoi and Tomita, '37), 48 mg. (Jung, '40), 8 to 16 mg. (Meulemans and de Haas, '38), 2.6 mg. (Hochberg et al., '43) and 2.9 to 10.0 mg. (Renner, '36).

The fact that fresh powdered whole milk averaged 12.5 mg. per liter vitamin C when reconstituted as compared with 5.8 mg. for market milk and 2.0 mg. for evaporated milk, shows that the powdered whole milk is capable of contributing an appreciable amount of vitamin C to the diet. The stability of vitamin C in powdered whole milk, even on long storage makes this product a good carrier of the vitamin.

SUMMARY

1. A comprehensive survey has been made to determine the vitamin C content of dairy products produced and sold commercially.

2. Pasteurized milk from consumers' homes and retail stores in a metropolitan area averaged 5.8 mg. and reconstituted evaporated milk from retail stores averaged 2.0 mg. total vitamin C per liter.

3. Fresh powdered whole milk averaged 12.5 mg. total vitamin C per liter reconstituted. Samples stored at room temperature, air packed, retained 88.7, 85.5 and 79.8% of their vitamin C content after 3, 6 and 12 months, respectively.

4. Raw milk entering the manufacturing plants averaged 17.1 mg. of reduced ascorbic acid per liter.

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STUDIES ON THE NUTRITIVE VALUE OF FISH PROTEINS

I. EVALUATION BY THE RAT GROWTH METHOD AND BY THE CANNON METHOD

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TWO FIGURES

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The normal supply of animal protein has been insufficient to meet the demands for the ever increasing requirements for proteins of high nutritional value. Therefore several attempts have been made to supply this deficiency with proteins of vegetable origin. In general, however, such proteins are usually considered to have a biological value lower than those of animal origin.

The fish supply offers a large reserve of available protein which according to the experiments of Holmes ('18) with human subjects has a high digestibility. These experiments were made with butterfish, Boston mackerel, grayfish and salmon.

Unfortunately, only a relatively small proportion of the total available fish catch is at present used for human consumption in the form of fresh, dried or canned products. The balance which is transformed into fish meal or other by-products is lost for direct human nutrition.

In order to provide a method whereby it would be possible to save the proteins present in the waste products, the pro-

teins were isolated from the easily perishable components and from the factors which make such by-products unpalatable. The present experiments were carried out in order to evaluate the biological activity of the proteins isolated commercially from the edible fish by-products.

The proteins of the three types of fishes most available commercially in Southern California waters, namely mackerel (Pneumatophorus jap. diego), sardine (Sardina coerulea), and tuna (Thunnus Thynnus and Sarda Chiliensis are the principal species), were tested for their biological value in growth experiments. In the case of mackerel protein, tests were also made using the Cannon procedure ('44). Further experiments on the nutritive value of mackerel protein are afforded by bioassays for vitamin A where this protein has been substituted for casein (Deuel, Hrubetz, Johnston, Rollman and Geiger, '45).

METHODS

The fish proteins used were prepared from edible fish muscle by isoelectric precipitation followed by purification by means of organic solvent extraction. The mackerel and sardine proteins were uniform white powders of high purity while the tuna preparation had a brown color and from the analyses appeared to be less pure. All of the fish proteins were without flavor. The casein used in control experiments was vitamin-test casein obtained from General Biochemicals, Inc. An analysis on a technical grade of casein is also included for comparison. The average composition of these proteins is given in table 1.

The amino acid composition of various types of fish has been reported earlier by numerous investigators and these data show the presence of all essential amino acids in desirable concentrations (Clark and Clough, '26; Bottinger and Baldwin, '40; Beach, Munks and Robinson, '43; Block and Bolling, '45). Some of these data were confirmed in our laboratory. The total amino acid analyses will be reported in a later communication.

Growth experiments

The growth experiments were performed on Sprague-Dawley rats. They were started at 21 days of age on the diets listed in table 2.

In each of the five series of tests, twelve rats were used. The rats in each series were equally divided into two groups each consisting of three male and three female rats. One group in each series received the fish protein diet under investiga-

TABLE 1

A comparative analysis of casein and purified fish proteins.

COMPONENT	CASEIN		FISH PROTEINS		
	Commercial	Vitamin-test	Mackerel	Sardine	Tuna
	%	%	%	%	%
Total N (Kjeldahl)	13.75	14.30	14.31	14.28	13.05
Protein (N × 6.25)	86.0	89.0	89.5	89.3	81.6
Water	7.0 (6.8)	4.1	7.0	6.2	6.6
Ash	2.2 (1.5–3.0)	1.9	0.8[1] (0.5–1.0)	0.8[1] (0.5–1.0)	0.8[1] (0.5–1.0)
Fat (Ether-extractable)	1.5 (1.0–2.0)	0.3	0.5[1]	0.5[1]	0.5[1]
Undetermined	3.3	4.3	2.2	3.2	10.5

[1] Approximate values.

tion while the second control group received the same level of casein. Litter mates were distributed equally between the two groups. Animals were fed ad libitum and in two groups the food consumption was determined.

Methods used in hypoproteinemia experiments

In general the procedures were similar to those outlined by Cannon et al. ('44). Large adult male rats from our stock colony weighing approximately 300 gm. at the start were

placed on the low protein diet employed by Cannon and continued on it for 95 days. They were then divided into three groups so that the average per cent of the original weight lost was identical in the three groups (43.8, 42.7 and 42.2%). Group I continued on the basal diet. In the diet for group II, 9% of the starch was replaced with casein and in group III 9% of the starch was replaced with the mackerel protein. At

TABLE 2

Composition of experimental diets.

DIETARY COMPONENTS	SERIES 1		SERIES 2		SERIES 3		SERIES 4		SERIES 5	
	Diet A	Diet B	Diet C	Diet D	Diet C	Diet E	Diet F	Diet G	Diet H	Diet I
Casein	9	..	12	..	12	..	15	..	21	..
Mackerel protein	..	..	..	12	..	..	..	..	..	21
Sardine protein	..	..	..	..	..	..	..	15	..	..
Tuna protein	..	9	..	..	..	12	..	..	..	..
Sucrose	74	74	71	71	71	71	68	68	62	62
Rice bran concentrate [1]	8	8	8	8	8	8	8	8	8	8
Cottonseed oil	4	4	4	4	4	4	4	4	4	4
Salt mixture [2]	4	4	4	4	4	4	4	4	4	4
Fish-liver oil [3]	1	1	1	1	1	1	1	1	1	1

To each 100 gm. of the different diets the following were added: riboflavin 1.5 mg., calcium pantothenate 3.0 mg., and chlorine chloride 50 mg.

[1] Supplied by the National Oil Products Co.

[2] U.S.P. XII no. 2.

[3] Contains 2000 I.U. vitamin A and 400 I.U. vitamin D per gram.

the end of 7 or 8 days, the rats were anesthetized with amytal, and a sample of approximately 0.1 ml. of blood was obtained from the tail. Plasma volume was then determined using Evans blue by the method as applied by Hechter ('45) using an interval for removal of the blood 5 minutes after the injection of the dye. Plasma protein was determined by the method of Mehl ('45) using a Klett-Summerson photoelectric colorimeter with a 540 filter and a conversion factor of 1400. Hemoglobin was estimated by the usual acid-hematin method on the Klett-Summerson colorimeter with a 420 filter.

RESULTS

The comparative results of the growth experiments where casein or fish protein was used are indicated in figures 1 and 2.

It is evident from these figures that in all cases better growth was obtained on the diets containing the fish proteins than on the diets containing the corresponding level of casein. It is especially striking that a greater growth rate should occur in the rat receiving the mackerel protein diet than in the casein rats when the proteins were fed at a level of 21%. It is

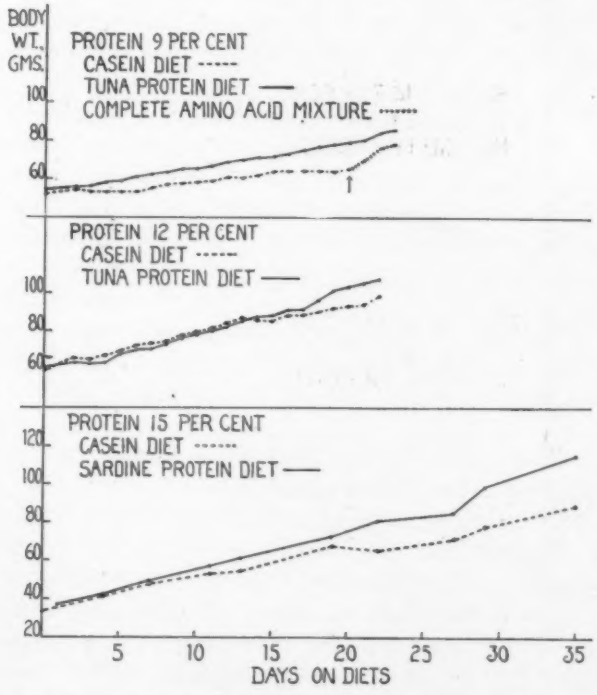

Fig. 1 The body weight of rats at various periods following weaning on casein or fish protein diets fed ad libitum. Tuna protein was used at 9% (diet B) in the experiments in the top graph, and at 12% (diet E) in those of the middle graph. Sardine protein (diet G) at 15% was the fish protein used in the lowest graph.

usually regarded that casein when fed at 18% affords an optimum growth.

The higher nutritive value of the tuna protein is suggested also by the fact that the total food consumed to give 1 gm. weight increase is much lower than for casein. In the rats receiving 9% of the tuna protein in the diet, an average of 157.6 gm. of food was consumed over the 24-day period and the average weight increase was 30.5 gm. The average ratio of food consumed to gain in weight was 5.2 (6.11, 5.88, 5.29, 5.14, 4.87, 4.35). In contrast to these values an average of 114.9

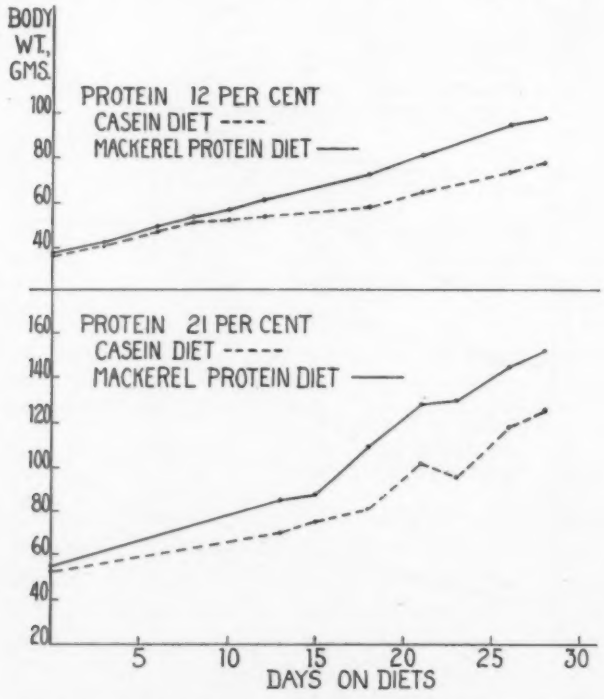

Fig. 2 The body weights of rats at various periods following weaning on casein or mackerel protein diets fed ad libitum. Diets C and D were used in the experiments given in the upper graph; diets H and I were used in the tests reported in the lower graph.

gm. of food were consumed in the control casein tests and the average gain in weight over the whole period was 8.0 gm. The mean ratio of food consumed to gain in weight was 14.3 (109.4, 28.1, 15.8, 11.8, 8.74, 8.20). It might be expected that at a higher protein level, the difference in the ratio would be less pronounced. In the experiments with 12% of protein in the food, the average ratio for the animals fed mackerel protein was 4.0 (4.11, 4.0, 4.0, 4.0, 3.97, 3.69) while that of the casein-fed rats was 4.8 (5.5, 5.1, 4.8, 4.4, 4.4. 4.4).

That the differences in growth in series 1 are significant is indicated by the fact that the maximum weight of any casein rat in all determinations after the tenth day is less than the average for the fish protein group for the corresponding period.

The data on the experiments on hypoproteinemic rats are summarized in table 3 which follows.

The weight increase after the casein diet was 20.2 gm. over what would have been expected had the rats continued on the basal diet, while the rise in weight of the rats receiving the mackerel protein on a similar basis amounted to 28.8 gm. The total protein eaten during the supplement period was highest with the fish proteins rats (9.71 gm.) and slightly lower with the animals receiving the casein diet (8.14 gm.) as compared with a protein intake of 1.24 gm. for the rats consuming the basal diet.

Hemoglobin showed a rise from the subnormal value in the control rats of 8.37% to 9.83 in the casein group and 10.42 in the mackerel protein group. On the other hand, the two proteins were equally effective in producing improvement in the serum-protein concentration. The increase from the marked hypoproteinemia of 3.02% (based on whole blood) to 3.70 and 3.66% for the casein and fish protein groups is highly significant from a statistical standpoint and indicates that a marked progress toward a return to a normal level had already occurred in 7 days.

The total plasma volumes were quite similar in the three groups. When based on the quantity per 100 gm. of body

TABLE 3

The comparative effect of casein (group II), mackerel protein (group III), and the basal low protein diet (group I) when fed over a 7-day period on the body weight, plasma volume, hemoglobin and plasma protein of male rats previously depleted by a prolonged low protein diet.

GROUP	NO. OF TESTS	AVERAGE BODY WT. IN DEPLETION PER.			SUPPLEMENTED PERIOD					BLOOD VALUES AFTER SUPPLEMENT			
					Average body weight		Change in body wt.	Total food eaten	Total protein eaten	Plasma vol.[1]		Hemoglobin [2]	Plasma proteins [2]
		Start	After 95 days	Loss	After 3 days	After 7 days				Per 100 gm.	Total		
		gm.	*gm.*	%	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*ml.*	*ml.*	%	%
I	14	286.0	161.0	43.8	157.7	156.3	− 4.7	69.5	1.24	6.25 (9)	10.25	8.37 ± 0.23 (6.80 − 9.82)	3.02 ± 0.08 (2.51 − 3.48)
II	16	285.0	163.3	42.7	168.2	178.8	+ 15.5	88.2	8.14	4.59 (6)	9.04	9.83 ± 0.40 (7.32 − 12.57) 3.20 [3]	3.70 ± 0.07 (3.19 − 4.42) 6.41 [3]
III	15	294.0	170.0	42.2	184.2	194.1	+ 24.1	104.8	9.71	5.21 (11)	10.42	10.42 ± 0.24 (8.39 − 11.80) 6.28 [3] 1.26 [5]	3.66 [4] ± 0.12 2.84 − 4.45) 4.44 [3] 0.29 [5]

[1] The values in parentheses are the number of experiments on which satisfactory determinations of blood volume were completed.

[2] Including the Standard Error of Mean calculated as follows: $\sqrt{\Sigma d^2/n} \;/\; \sqrt{n}$.

[3] Mean Difference/Standard Error of Mean Differences when group is compared with group I. When this ratio exceeds 3.00, it is considered that the differences are statistically significant.

[4] This average based on only 13 determinations.

[5] Comparison of M.D./S.E.M.D. between groups II and III.

weight, the mean was highest in the basal control group (6.25), intermediate in the fish protein group (5.21) and lowest in the casein rats (4.59). The fact that the discrepancies become small when based on the total plasma volume without reference to body weight would indicate that the rapid variations in weight occurring during the supplement period were not accompanied by similar rapid adjustments in plasma volume.

Although no values of plasma volume are reported on control rats on a diet containing an adequate protein level throughout the depletion period, they are quite similar to those obtained with normal rats of our stock colony by Hechter ('45). Moreover, no alterations in value are evident when protein is administered for a week. On the other hand, Metcoff, Favour and Stare ('45) have indicated a significant decrease in plasma and blood volumes in acute protein deficiency.

DISCUSSION

All the experimental evidence is in agreement that the mixed mackerel muscle proteins have a considerably higher biological value than casein. Tuna proteins when fed at 9 and 12% and sardine proteins at 15% also gave considerably more rapid growth in weanling rats than diets containing similar amounts of casein. One should probably expect such a superiority for a mixed protein over a homogenous protein as casein as the deficiencies of one protein may to a considerable extent be supplemented by the other proteins.

The evaluation of biological activity by the Cannon method may well prove to be a more quantitative procedure than the usual growth method. It would appear to the authors that the factor for comparison should not be the weight increase over the 7-day period but rather this value plus the weight deficit of the control unsupplemented rats during the same interval.

The ratio of biological activity of fish protein to casein based on weight increases of the hypoproteinemic rats over a 7-day period would be on the first basis 1.55 (24.1 : 15.5) while by the second method it would be 1.43 (28.8 : 20.2).

[5] Comparison of M.D./S.E.M.D. between groups 11 and 12.

On the basis of hemoglobin increase, it would also appear that the mackerel protein was somewhat superior to casein. The hemoglobin in the control group (8.37%) indicates that the prolonged protein-low diet had resulted in a severe anemia. This level was increased to 9.83 in the rats which received casein and to 10.42% for those animals which received the fish protein diet. Although the hemoglobin averaged considerably higher in the fish protein series than in the casein group, the differences are not significant from a statistical standpoint because of the relatively high variability in the individual experiments.

The plasma protein figure for the control rats is distinctly subnormal. Casein and mackerel protein were both equally effective in causing an increase in this value, the averages after the supplement period being 3.70 and 3.66%, respectively.

The fact that mackerel protein may be superior to casein in hemoglobin formation while it shows no superiority in causing the regeneration of the serum proteins may be explained in several ways. Either its amino acid makeup favors hemoglobin synthesis or as Whipple ('42) suggests, hemoglobin regeneration has a priority over the formation of plasma protein even though the amino acid distribution may be less suited to the former. Further comparative tests on rats in which each of these factors is varied separately should answer this question. It would also be of considerable interest to compare the biological efficiency of the single proteins making up the mixtures of the fish proteins used here.

SUMMARY

The mixed proteins of mackerel, sardine and tuna muscle were shown to afford superior growth to casein in weanling rats when fed at levels which give suboptimal growth with casein.

The superiority of mackerel protein is further attested by the fact that it causes greater recovery in weight and a more pronounced stimulation in hemoglobin regeneration than casein in rats rendered hypoproteinemic and tested by the

method of Cannon. Mackerel protein proved to have a potency equal to casein in causing a regeneration of plasma protein.

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STUDIES ON THE NUTRITIVE VALUE OF FISH PROTEINS

II. THE USE OF MACKEREL PROTEIN IN THE BIOASSAY TEST FOR VITAMIN A

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Casein has usually been the protein of choice which is incorporated in the diets used in the bioassay tests to determine vitamin A. In fact, it is prescribed in the U.S.P. diet to be used for vitamin A assay. This is presumably because of its ready availability and the ease with which it may be rendered vitamin A-free. Moreover, it is a protein which possesses a high nutritive value when fed at a level of 18% in the diet.

Although it is well known that growth cannot be brought about when any single essential component of the diet is absent, the present experiments afford an opportunity to determine the effect of a protein other than casein when fed as a constituent of a diet limited in vitamin A. The protein used in these experiments was mackerel protein which possesses a biological activity considerably in excess of casein (Deuel et al., '45). Moreover, by its method of preparation it would be expected to be free of vitamin A and this was also indicated by spectrophotometric analysis. Likewise, it is a protein potentially available in unlimited amounts.

METHODS AND RESULTS

The bioassay tests were carried out by the U.S.P. XII method except that the basal diet in one group was modified by

the substitution of mackerel protein for casein. In series II where the protein was in some cases reduced to 9%, it was replaced by the addition of a corresponding amount of starch. In series III rats were started on the bioassays as soon as they were sufficiently depleted when the average length of depletion period was about 17 days instead of 20 days prescribed as the minimum in the U.S.P. method.

Three series of tests were made on 222 rats. Series I and II were conducted on rats from the U.S.C. colony and series III with animals of the Sprague-Dawley strain. In series II, the animals were started on the respective diets at weaning while in the other tests, the experimental diets were employed only during the assay period. The vitamin A supplements were made from U.S.P. Reference cod-liver oil. They were made up in cottonseed oil with 5 mg. of alpha tocopherol added per milliliter. A total of 0.1 ml. of the supplements was administered daily. The negative controls in each series were given 0.1 ml. of the cottonseed oil-tocopherol mixture daily. Litter mates were distributed between the various groups. Animals were considered sufficiently depleted to start on the assay period when their weight was stationary for 5 days or after a shorter period if a marked drop in weight (more than 6 gm.) occurred. Food was given ad libitum. The amount of the food consumption was determined in the second series. The results of the tests are summarized in table 1.

DISCUSSION

A commercial mackerel protein, prepared by isoelectric precipitation followed by purification by solvent extraction, would appear to be perfectly adequate for use in the bioassay test for vitamin A in place of casein. In the first place, it is extremely low in vitamin A so that depletion occurs when it is fed from weaning in a similar period to casein (16.7 days for the fish protein compared with 17.6 days for the control casein rats in our tests). Also, in all cases as large a proportion of the negative controls died during the tests with the fish protein

TABLE 1
The effect of different dietary proteins on the growth of rats receiving given amounts of vitamin A

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The effect of different dietary proteins on the growth of rats receiving given amounts of vitamin A.

SERIES NO.	BASAL DIET			VITAMIN A ADMINISTERED DAILY	NUMBER OF EXPERIMENTS		ASSAY PERIOD						
	Protein	Per cent protein	Per cent cystine		Male rats	Female rats	Body weight [1]		Av. weight increase			Av. daily food intake	Average day of death [2]
							Start	End	Male	Female	Total [1]		
USC rats													
				I.U.			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
I	Casein	18	..	1.5	5	7	85.0	103.7	10.2	24.4	17.3	...	15
											(11)		(1)
	Mackerel	18	..	1.5	5	7	87.8	106.6	18.4	19.5	19.0	...	14.7
											(9)		(3)
	Casein	18	..	3.5	3	6	91.8	141.6	60.5	39.3	49.9	...	..
	Mackerel	18	..	3.5	4	5	86.6	139.2	62.0	43.3	52.6	...	..
	Casein	18	..	0.0	7	6	89.5	....	...	...	— 24.0	...	19.3
											(2)		(11)
	Mackerel	18	..	0.0	6	7	81.7	....	...	...	— 14.0		19.6
											(3)		(10)
II	Casein	18	..	2.0	6	8	99.7	148.6	56.6	41.2	48.9	8.91	..
	Mackerel	18	..	2.0	3	7	92.1	145.3	62.0	44.3	53.2	9.57	
	Casein	9	..	2.0	3	5	100.5	147.1	52.0	41.3	46.6	10.02	..
	Mackerel	9	..	2.0	7	6	88.4	141.6	62.6	43.7	53.2	9.52	..
	Casein	9	1	2.0	7	6	102.6	153.3	56.0	45.3	50.7	9.40	..
	Casein	9	1	0.0	7	6	98.8	....	...	...	— 0.9	5.68	21.2
											(5)		(8)
	Mackerel	18	0	0.0	8	6	101.4	....	...	...	— 14.2	5.28	18.0
											(6)		(8)
Sprague-Dawley rats													
III	Casein	18	..	1.2	5	6	69.6	107.8	43.0	33.3	38.2	...	..
	Mackerel	18	..	1.2	5	7	69.4	123.9	59.0	50.0	54.5	...	..
	Casein	18	..	3.5	4	7	71.2	136.0	68.5	50.4	59.4	...	..
	Mackerel	18	..	3.5	5	7	71.8	149.8	86.4	69.5	78.0	...	..
	Casein	18	..	0.0	5	6	74.5	....	..	...	...	...	18.3
													(11)
	Mackerel	18	..	0.0	4	8	69.2	....	...		...	...	14.0
													(12)

The figures in parentheses indicate the number of experiments in the average.

[1] The averages of the males and females are weighed equally.

[2] This value is for the decedents only and does not include those rats which survived the 28-day test period.

as with the casein and the average day of death for the decedents was slightly less than for casein.

The extent of growth during the 28-day bioassay period when vitamin A was given was significantly greater with the mackerel protein in the experiments where Sprague-Dawley rats were employed. The growth of such rats receiving the fish protein exceeded that of the rats on the standard casein diet by 43 and 31% where vitamin A was administered at levels of 1.2 and 3.5 I.U., respectively. On the other hand, with the U.S.C. strain the differences in growth on the two proteins were not significant although in each case the increase in weight with the group receiving the mackerel protein somewhat exceeds that of those receiving casein. When 1% of cystine was added to the diet containing 9% casein (Mendel, '15), the growth was slightly though not significantly increased over that obtained where this amino acid was omitted. This addition had no effect on the survival time or on the proportion of rats which died in the negative control group where no vitamin A was administered. The increased growth response of the mackerel protein over casein obtained with series III is supported by the results of the earlier experiments where a more pronounced growth was shown to obtain with mackerel protein even when the proteins were fed in a complete diet at a level of 21% to weanling rats (Deuel et al., '45).

These experiments are of interest in relation to the recent report of Dye, Bateman and Porter ('45) who were unable to demonstrate any increased growth response to vitamin A when casein was fed at levels of 9, 18 and 36%. However, these experiments are not directly comparable to ours inasmuch as paired feeding was employed while in the present tests the animals were fed ad libitum. It is also true that although greater growth might occur on the higher protein level when the net caloric intake is identical with that on the lower level of protein, such results can only be attained when the food consumption is increased sufficiently with the high protein diets to compensate for the larger loss due to the specific dynamic action of the protein. It would appear if an

identical growth occurs on isocaloric diets with low and high protein that a greater efficiency characterizes the latter case. A smaller net balance of calories is available in the latter case after that required for basal metabolism and specific dynamic action has been deducted from the total.

It is evident that not only the caloric intake (Muelder and Kelly, '41) but also the quality of the protein may influence the growth response to given amounts of vitamin A. If limited quantities of food are taken as may be the case on diets where minimum amounts of vitamin A are available, there is less chance that a protein deficiency migh occur when a protein of higher biological value than casein is employed. In addition, other growth factors are involved inasmuch as it has been shown that the amount of growth hormone available will influence such a growth response (Ershoff and Deuel, '45) when administered with limited amounts of vitamin A.

SUMMARY

A commercially prepared mackerel protein powder has been shown to be a satisfactory protein to use in bioassay experiments for the determination of vitamin A.

This protein contained no detectable quantity of vitamin A as indicated by the fact that the average number of rats in the control groups surviving the 28-day test period as well as the average survival period of the decedents compare favorably with the casein tests.

When uniform doses of vitamin A were administered daily, greater growth was obtained in rats receiving the fish protein diet than in litter mates which were fed the corresponding casein diet. The differences were statistically significant in the Sprague-Dawley rats but not in those of the U.S.C. strain. This would indicate that greater growth may result with limited amounts of vitamin A when a protein of higher nutritive value than casein is employed.

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THE AVAILABILITY OF WHEAT BRAN PHOSPHORUS FOR THE RAT [1]

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Patten and Hart ('04) showed that practically all of the phosphorus of wheat bran is in the form of salts of phytic acid. Hart, McCollum and Fuller ('09) demonstrated that wheat bran could serve as the main source of available phosphorus for the skeletal development of young swine. Pigs on a low phosphorus basal ration supplemented with wheat bran produced thigh bones with an ash content of 53 to 54%, while those receiving precipitated calcium phosphate as the phosphorus supplement, and at the same phosphorus level, produced thigh bones with an ash content of 46 to 55%. On the basal ration the ash content was 31 to 33%. Approximately 81% of the total phosphorus of the bran ration was phytic acid phosphorus. The pigs had access to sunlight.

Hart and coworkers ('27) observed that cattle in a phosphorus deficient area of Wisconsin were completely protected from rickets by the addition to the ration of either wheat bran or bone meal. Both products served to contribute phosphorus to the ration. These animals also had access to direct sunlight during the experimental period.

All of the above studies pointed toward the availability of the phytic acid phosphorus of wheat bran for swine and cattle,

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at least where this product was fed under practical conditions with sunlight available. Since these observations were made, there has accumulated definite evidence that in the absence of vitamin D, the phosphorus of isolated phytic acid is poorly available to the rat (Krieger, Bunkfeldt, and Steenbock, '40). However, it was also shown by Krieger and coworkers ('40, '41) that in the presence of vitamin D there is a marked improvement in the utilization of isolated phytic acid phosphorus. In studies with chicks, Lowe, Steenbock and Krieger ('39) and McGinnis, Norris, and Heuser ('44) showed that the phosphorus of cereals and legumes was poorly available in the absence of vitamin D, and the latter group found phytin-phosphorus nearly as available as inorganic phosphorus in the presence of 160 A.O.A.C. units of vitamin D per 100 gm. of diet.

McCance and Widdowson ('35) have pointed out that because approximately 40 to 70% of the phosphorus of cereal grains is in the form of phytic acid, cereals and cereal products are a poor source of available phosphorus for man. From their experiments with human subjects they concluded that when cereals constitute the largest part of the diet, the low availability of phytin phosphorus can become a serious matter.

It has been suggested by Hart et al. ('09), Lowe and Steenbock ('36), and Singsen and Mitchell ('44) that ingestion of the enzyme phytase with the food may be necessary for the efficient utilization of phytin phosphorus. Thus the observation of Krieger, Bunkfeldt and Steenbock ('40) that the phosphorus of isolated phytic acid fed in a purified diet is of poor availability might be explained by a lack of dietary source of phytase. Likewise, the low availability of the phosphorus of cereals observed by Bruce and Callow ('34) for rats and by McCance and Widdowson ('35) in man might be similarly related to an inadequate dietary source of phytase since cooked cereals were used in part. Singsen and Mitchell ('44) found that the efficiency of the chick in utilizing the phytin phosphorus of soybean meal was satisfactory when field cured alf-

alfa meal was included in the ration as a source of phytase. Dehydrated alfalfa meal was not effective, presumably because the phytase had been destroyed during processing. However, the validity of this interpretation is open to question since the field cured alfalfa hay supplied extra vitamin D as well as phytase. A feeding oil furnished vitamin D at a level calculated to be 100 A.O.A.C. units per 100 gm. of ration, which may have been inadequate. Spitzer and Phillips ('45a, b) concluded that a dietary source of phytase was not essential for the utilization of the phytin phosphorus of soybean meal by the rat.

With these facts in mind, two experiments were made with rats to study the effect of dietary phytase and vitamin D on the availability of the phosphorus of wheat bran.

EXPERIMENTAL

The basal ration was similar to the low phosphorus diet developed by Schneider and Steenbock ('39) and had the following composition: cerelose (glucose monohydrate) 50, dextrin 20, fibrin [2] 20, phosphorus-free salts 4, cottonseed oil (plus 100 μg. β-carotene per gram) 5, and 1 to 20 liver concentrate 1. Water soluble vitamins were incorporated in the ration so that every 100 gm. of ration contained 0.5 mg. thiamine, 0.5 mg. riboflavin, 0.6 mg. niacin, 0.6 mg. pyridoxine, 5.0 mg. calcium pantothenate, 30.0 mg. p-aminobenzoic acid, 100.0 mg. inositol, and 250.0 mg. choline.

The phosphorus-free salts were prepared by substituting calcium lactate and an equimolecular mixture of KCl and $KHCO_3$ for the $CaHPO_4$ and K_2HPO_4 of salts IV (Hegsted, Mills, Elvehjem and Hart, '41). The substitutions were equimolecular with respect to Ca and K.

The raw wheat bran as well as the commercial bran cereal [3] contained from 1.39 to 1.40 gm. of phosphorus [4] per 100 gm.

[2] The use of fibrin in low P rations was suggested by Jones ('38).

[3] Kellogg's All-Bran.

[4] Phosphorus determinations were made by the method of Fiske and Subbarow ('25). Measurements were made in the Evelyn colorimeter.

and was incorporated in the phosphorus-free basal ration in place of cerelose at a level of 20% to furnish 0.28 gm. of phosphorus per 100 gm. of ration. This made the test diets identical in phosphorus content with a control ration in which 4% of salts IV were substituted for the phosphorus-free salts. A low phosphorus control ration was made by replacing the test bran with a washed, phosphorus-free bran. In this way, an approximate equivalence in fiber was maintained. The washed bran was prepared by incubating raw wheat bran with water at 37°C. for 24 hours to allow the phytase to liberate inorganic phosphorus. After filtering, washing and drying, the washed bran contained 0.02% of phosphorus. Consequently it contributed 0.004 gm. of phosphorus per 100 gm. of ration at the 20% level. The fibrin contributed approximately 0.02 gm. of phosphorus per 100 gm. of ration.

All of the rations contained 0.60% of calcium. The 4% of salts IV as well as the phosphorus-free salts contributed 0.59 gm. of calcium to each 100 gm. of ration and the bran added 0.01 gm. A calcium to phosphorus ratio of 2 existed in all experimental diets.

Male albino rats of the Sprague-Dawley strain 32 days of age were divided into groups of 4 with an average weight of 68 gm. Each rat was housed in an individual raised bottom cage. The daily allotment of food was equalized for all rats except those fed the phosphorus-free ration. After 4 weeks the femurs of each animal were removed and the ash of individual bones was determined by the official A.O.A.C. procedure for the rat.

In the first experiment the effect of vitamin D on the utilization of the phosphorus of the phytic acid of wheat bran was determined. A prepared bran cereal, Kellogg's All-bran, was chosen for the test because it represents a commercial product high in phytic acid with no phytase activity.

The following rations were fed to groups of four rats:

Group 1. Low phosphorus control, no vitamin D, washed bran, phosphorus-free salts.

Group 2. Inorganic phosphorus control, 250 I.U. of vitamin D [5] per rat each week, salts IV, washed bran.

Group 3. Test bran without vitamin D, phosphorus-free salts.

Group 4. Test bran plus 70 I.U. of vitamin D [6] per 100 gm. of ration, phosphorus-free salts.

Group 5. Test bran plus 140 I.U. of vitamin D [6] per 100 gm. of ration, phosphorus-free salts.

Group 6. Test bran plus 210 I.U. of vitamin D [6] per 100 gm. of ration, phosphorus-free salts.

Group 7. Test bran plus 280 I.U. of vitamin D [6] per 100 gm. of ration, phosphorus-free salts.

The second experiment was designed to study the effect of a dietary source of the phosphate liberating enzyme, phytase, on the utilization of phytic acid in wheat bran. The sample of raw wheat bran (also used in the first experiment for the preparation of the washed bran) was found to contain an acid phosphatase. A portion of the bran was autoclaved at 15 pounds pressure for 1 hour to destroy enzyme activity. Rations were made from these two samples of bran similar in composition and phosphorus content to those fed in the first experiment. The plan of the test was as follows (a third control ration, group 2, was included in this experiment):

Group 1. Low phosphorus control, no vitamin D, phosphorus-free salts, washed bran.

Group 2. Inorganic phosphorus control, no vitamin D, salts IV, washed bran.

Group 3. Inorganic phosphorus control, 250 I.U. vitamin D per rat each week,[5] salts IV, washed bran (repetition of group 2 in experiment 1).

Group 4. Raw wheat bran, no vitamin D, phosphorus-free salts.

Group 5. Raw wheat bran with 250 I.U. of vitamin D per rat each week,[5] phosphorus-free salts.

[5] Drisdol. Winthrop Chemical Co. Irradiated ergosterol in propylene glycol. This supplement was given by pipette.

[6] Irradiated ergosterol was incorporated in the ration.

Group 6. Autoclaved wheat bran, no vitamin D, phosphorus-free salts.

Group 7. Autoclaved wheat bran plus 250 I.U. of vitamin D per rat each week,[5] phosphorus-free salts.

The determination of phytase activity was made according to the method of Spitzer and Phillips ('45b). A 50-mg. sample of raw wheat bran liberated 110 μg. of inorganic phosphorus when incubated for 2 hours with a sodium phytase substrate containing 230 μg. of phosphorus. This value was corrected for the inorganic phosphorus present at zero time and for the phosphorus liberated during the 2 hours from substrates carried in the enzyme preparation. A 200-mg. sample of either the autoclaved bran or the commercial bran cereal showed no phytase activity.

RESULTS

The growth data together with the per cent of ash of the femurs are given in tables 1 and 2. The rats on the low phosphorus control diet grew slowly and reached a maximum

TABLE 1

Record of growth and bone ash in the first experiment.

Source of Phosphorus	None	In-organic	Commercial bran cereal				
Vitamin D/100 gm. Ration	None	Excess	None	70 I.U.	140 I.U.	210 I.U.	280 I.U.
Group Number	1	2	3	4	5	6	7
Average initial weight	69	68	70	66	67	67	67
Average final (4 wks.) weight	110	156	154	153	162	150	149
Average bone ash	24.7	57.4	36.4	46.3	50.6	49.2	48.3
Mean deviation	1.1	0.57	1.7	1.5	1.3	1.1	1.8

weight between the third and fourth week when weight losses became evident. Food consumption dropped to about 6 gm. per rat per day during the fourth week. Those rats fed rations containing phosphorus grew 20 to 25 gm. a week and consumed up to 16 gm. of ration a day by the end of the ex-

periment. The food allotment was limited in both studies to the amount eaten by the rats fed the raw bran ration without vitamin D.

In the first experiment a bone ash of 24.7% was found for animals fed the phosphorus-free ration. The addition of 0.28% of inorganic phosphorus and adequate vitamin D to this ration allowed development of bones of 57.4% ash. These findings were repeated in the second experiment, and in addition it was found that even in the absence of vitamin D, rats fed this ration developed femurs of 54.4% ash content. However, when the 0.28% of phosphorus was contributed to the

TABLE 2

Record of growth and bone ash in the second experiment.

Source of Phosphorus	None	Inorganic		Raw bran		Cooked bran	
Vitamin D	None	None	Excess	None	Excess	None	Excess
Group Number	1	2	3	4	5	6	7
Average initial weight	68	69	68	68	69	68	68
Average final (4 wks.) weight	110	169	176	163	165	166	165
Average bone ash	21.4	54.4	56.7	32.0	48.8	38.9	50.4
Mean deviation	0.90	1.5	1.0	1.2	1.7	3.3	1.0

ration by the commercial bran without added vitamin D, a bone ash of 36.4% resulted. This value was increased to about 50% by the addition of vitamin D to the ration.

In the presence of vitamin D, both the autoclaved and the raw wheat bran gave a bone ash picture similar to that obtained with the commercial bran. In the absence of vitamin D, the bone ash of rats fed raw bran was 32.0%; values of 38.9 and 36.4% resulted from the same bran cooked and the commercial bran cereal.

DISCUSSION

The presence or absence of the enzyme phytase in the diet had no apparent effect on the availability of phytin phosphorus. In fact, the bone ash values of the rats fed the raw

bran supplement, which contained phytase, were not as great as those of the rats receiving inactivated bran. This indicates a partial hydrolysis of phytin during cooking. It is probable that the phosphorus splitting enzymes elaborated by the intestinal mucosa (Spitzer and Phillips, '45b) and by the flora of the gastro-intestinal tract are the critical means by which phytin is hydrolyzed in the intestine of the rat.

The absolute amounts of calcium and phosphorus in these rations were adequate (Nicolaysen, '37) and a calcium to phosphorus ratio of 2 was maintained. Since about 85% of the phosphorus in these diets was in the form of phytin (Patten and Hart, '04), the importance of vitamin D is clearly demonstrated. The bone ash values of the rats receiving phytin phosphorus and adequate vitamin D were only 4 to 7% lower than those of the rats receiving the inorganic phosphorus supplement. The low availability of the phytin phosphorus of cereals has been overemphasized in the literature. As a result it is often not realized that satisfactory bone development is possible with diets carrying only a normal amount of phosphorus mainly in the form of phytin.

These data support the results of Krieger et al. ('40) who found that vitamin D aided in the utilization of the phosphorus of isolated phytic acid by the rat, and of McGinnis et al. ('44) who showed the importance of vitamin D for the utilization of phosphorus in cereals and legumes by the chick. Further, it appears from our data that there may a direct relationship between the level of phytic acid phosphorus in the ration and the vitamin D requirement.

SUMMARY

1. A study of the availability of phytin phosphorus was made with the rat using diets of normal calcium and phosphorus levels. About 85% of the dietary phosphorus was in the form of phytic acid and its salts, as carried by wheat bran.

2. In the absence of vitamin D, the phytin phosphorus of wheat bran was poorly utilized. An adequate intake of this

vitamin increased the utilization nearly to that of inorganic phosphorus as measured by bone ash.

3. The presence or absence of phosphorus splitting enzymes in the diets did not alter the apparent availability of phytin phosphorus.

NOTE

After this paper was prepared for The Journal of Nutrition an article by Singsen and Mitchell appeared (Poultry Sci., vol. 24, p. 479, 1945) in which is found confirmation of the idea that the availability of phytin phosphorus is related to the amount of vitamin D in the ration.

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RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

I. BY THE GROWING ALBINO RAT [1]

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Fat, as a dietary component, is characterized by several unique capacities. In addition to being the nutrient of maximum energy value it confers palatability and "staying quality" to diets. It serves important purposes as a carrier of vitamins A, D and E, and provides the apparently essential linoleic or linolenic acids. It contributes to the composition of tissue lipids; it insulates, supports and cushions vital organs; as a nutritive reserve it insures the availability of energy as needed; and it diminishes the energy expense of utilization of the nutrients with which it is associated.

In spite of the existing knowledge of these functions of the fats, however, the student of nutrition has still to deal with a popular understanding that, for practical purposes, fat and carbohydrate may be regarded as interchangeable in accord with their metabolizable energy values, fat therefore being approximately 2.25 times as potent as carbohydrate for the same purposes.

During recent years a volume of evidence has accumulated as to the utilization of fats and carbohydrates, individually and in combination with other nutrients, and especially as affected by associated vitamins, which emphasizes the complication of conditions which determine the relative values of

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these nutrients, and warrants the conception of the facts in this matter as determined, in large part at least, by the relative extent to which the requirements of fat and of carbohydrate utilization are satisfied by any nutritive ensemble in question.

It thus becomes clear that the only logical background for the determination of the interrelations of fats and carbohydrates in nutrition is one which affords optimum conditions for the utilization of each.

However, it is still necessary, for purposes of guidance in nutritional affairs, to observe the facts in this matter as they are encountered under conditions of dietary practice.

Forbes and Swift ('44) recently demonstrated that fat decreases the energy expense of utilization of the food protein and carbohydrate with which it is combined. This paper and the following now report the results of two further studies of the functions of fat, one with growing and the other with mature albino rats as subjects, both groups having received the same diets containing 2, 5, 10 and 30% of fat, respectively; these diets were rendered isocaloric by compensating adjustment of carbohydrate contents, and so compounded and fed that each supplied gross energy, protein, and vitamins in the same proportion.

The first of these experiments, with weanling rats as subjects, was conducted by the so-called body balance method of this laboratory in accord with which the rats are allowed normal freedom of movement, and in which a single measurement of the heat production for a period of 70 days is made by subtraction of the energy of the excreta and of the body increase from the gross energy of the food.

In connection with this procedure values were determined for the digestibility of the diets, and for the retention of energy and of nitrogen, the observed differences in nutrient utilization being considered as resultants of the differences in the metabolism of energy and protein, in the dynamic effects of the diets, and in the physical activity of the subjects.

The second of these experiments, with mature rats as subjects, was conducted by the open-circuit Haldane procedure, with voluntary activity excluded. The heat increment or dynamic effect of the food was determined as the difference in the amounts of heat produced from a basal and a supplemented diet, both of the same composition, and containing 2, 5, 10 or 30% of fat.

The first of these experiments, with weanling rats as subjects, is discussed in the present, and the second, with mature rats as subjects, in the following paper.

EXPERIMENTAL

The diets [2] were compounded as indicated in table 1. The amounts of vitamins incorporated in the diets were determined in consideration of evidence presented by Burr and

TABLE 1

General composition of diets.

	DIET NO. 1	DIET NO. 2	DIET NO. 3	DIET NO. 4
	%	%	%	%
Salt mixture [1]	4.00	4.00	4.00	4.00
Corn oil [2]	2.00	2.00	2.00	2.00
Protein mixture [3]	31.40	32.82	35.19	44.67
Carbohydrate mixture [4]	62.60	58.18	50.81	21.33
Lard	0.00	3.00	8.00	28.00
Calories per gm.	4.058	4.241	4.548	5.773
Isocaloric factors	1.0000	.9567	.8922	.7029

Vitamin supplements were added in the following amounts per kg. of Diet 1, and to equicaloric quantities of the other diets: carotene 6.0 mg., thiamine-HCl 5.0 mg., riboflavin 5.0 mg., pyridoxine-HCl 6.25 mg., niacin 6.25 mg., calcium pantothenate 50 mg., choline chloride 400 mg., para-aminobenzoic acid 95 mg., 2-methyl-1,4 naphthoquinone 2.5 mg., alpha-tocopherol 100 mg., and inositol 2.0 gm. Vitamin D was supplied in irradiated yeast in the amount of 47.1 gm. per kg. of the 2% fat diet and of isocaloric quantities of the other diets.

[1] U.S.P. XII no. 2.

[2] Considered to furnish sufficient essential fatty acid.

[3] Casein 50%, skim milk powder 25%, irradiated yeast 15%, brewer's yeast 10%. Mixture contained 10.19% N.

[4] Corn starch 34%, sucrose 33%, dextrin 22%, and dextrose (cerelose) 11%.

[2] The authors gratefully acknowledge the supply of the vitamin components of the diets by Merck and Company.

Burr ('29), Evans and Emerson ('43), Griffith and Farris ('42), Martin ('39), and Rosenberg ('42).

Diet no. 1 was made to contain 20% of protein. The other three diets were compounded by means of an algebraic computation based on the nitrogen and energy contents of the non-nitrogeneous nutrients to such effect that the ratio of the energy of the protein to the energy of the diet as a whole was constant in all four diets. It was then possible, by the use of the so-called isocaloric factors in table 1, to feed the four diets so as to supply the same amounts of protein and energy to each rat.

TABLE 2

Constituents of diets of different fat and different carbohydrate contents which supply the same quantities of protein and of energy.

DIET NO.	APPROXIMATELY ISOCALORIC QUANTITIES OF DIETS	PROTEIN		FAT [1]		CARBOHYDRATE [2]		ENERGY	
	gm.	%	*gm.*	%	*gm.*	%	*gm.*	*cal./gm.*	*Cal.*
1	10.00	20.00	2.000	2.07	.207	67.78	6.788	4058	40.58
2	9.57	20.90	2.000	5.07	.485	63.68	6.654	4241	40.57
3	8.92	22.41	2.000	10.08	.899	57.66	6.464	4548	40.58
4	7.03	28.45	2.000	30.10	2.115	32.70	4.651	5773	40.58

[1] Fat added as corn oil and lard plus a very small amount in the protein mixture.
[2] Carbohydrate added as carbohydrate mixture and 22.75% of the protein mixture.

Table 2 gives the quantities of the diets required to supply protein and energy each at a constant rate; and also the protein, fat, carbohydrate and energy contents of isocaloric portions of the diets.

The proportions of fat to carbohydrate in diets nos. 1, 2, 3 and 4 were 1 : 32.8, 1 : 13.7, 1 : 7.19 and 1 : 2.20, respectively; and the reciprocal proportions of carbohydrate to fat in diets nos. 1, 2, 3 and 4, were 1 : .031, 1 : .073, 1 : .139, and 1 : .455, respectively.

While these ratios constitute the really significant bases for conclusions from the investigation, the diets will be designated, for convenience, by their approximate fat contents — to wit, 2, 5, 10 and 30%.

The experimental subjects were ten litter-fours of weanling albino rats subdivided in a manner providing four groups of ten individuals, each such group containing one rat from each of ten litters.

The rats were fed once a day, the amount given to each rat of a litter-four being determined by the individual of the four which ate the smallest amount of food. By this method of food control the caloric and the nitrogen intakes of each group of ten rats were maintained identical. The palatability of the diets increased in the order of their fat content, there being a total of thirty-two feed refusals by the rats receiving the 2% fat diet and only three by the rats receiving the 30% fat diet.

The urines were collected daily, and two aliquots were prepared, one preserved with 25 ml. of 1.7 Sp. Gr. H_2SO_4 per 5-pint bottle for the determination of nitrogen, while the other was dried for the determination of energy by means of the bomb calorimeter. The feces were also collected daily, and after drying at 50°C., were allowed to come into moisture equilibrium with the air before being weighed and ground for analysis.

This experiment was conducted at an environmental temperature thermostatically controlled at 29.5°C.

The bodies of the rats were cut up with shears, and were prepared for extraction by drying over sulphuric acid in vacuum desiccators at room temperature. After extraction with ether the residues were ground in an Excelsior mill, which reduced them to a condition sufficiently finely divided for sampling and determination of nitrogen and energy.

Table 3 gives the average weekly live weights of the four groups of ten rats, each of which groups received one of the experimental diets during the 10 weeks of the experiment.

An inspection of these average weekly weights for ten rats shows that the capacities of the diets to produce gain in weight were in the increasing order of their fat contents. During the third to the ninth week the rats which received diet no. 4, containing 30% of fat, were 3, 4, 7, 11, 14 and 16

gm. heavier than the rats which received the same amounts of energy in diet no. 1, containing 2% of fat. Since each rat of a litter-four was held to the same caloric intake, the rats were in effect penalized in accord with the amount that they grew, by virtue of the fact that the greater the growth the greater is the maintenance requirement. The observed differences in gain in weight produced by the four diets, therefore, were smaller than they should have been if the basis of comparison had been perfectly equitable.

TABLE 3

Average liveweights of rats during 10 weeks on isocaloric quantities of diets containing 2, 5, 10 and 30%, respectively, of fat.

DIET NO.	FAT CONTENT OF DIET	INITIAL BODY WEIGHT	WEEK NUMBER									
			1	2	3	4	5	6	7	8	9	10
	%	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	2	48.5	63	93	114	134	154	169	184	200	210	220
2	5	48.2	63	93	115	136	157	174	191	207	220	231
3	10	47.2	62	94	117	136	158	176	194	210	225	235
4	30	47.7	62	93	117	138	161	180	198	216	230	239

The average amounts of food and food nitrogen eaten per rat during 70 days by each of the groups of ten, and the average initial and final weights of body nitrogen and of body fat, are set forth in table 4. The gains in body nitrogen and body fat increased in the order of the increasing fat content of the diets, except that the increase in the body fat of the rats which received the 30% fat diet was not greater than that of the rats which received the 10% fat diet.

In regard to the cause of the increased fat contents of the rat bodies with increase in the fat content of the diets — this could logically be due either to a fundamental advantage in favor of the energy expense of utilization of fat, or to a difference in the effects of the dietary conditions, especially as to vitamin contents, as affecting the utilization of fat and carbohydrate. The intake of vitamins, however, is believed to have been adequate.

It is a fact, however, that the body residues from the ether extraction in the coarsely cut-up condition were not re-extracted after grinding. The weights of body fat, therefore, were not of the most critical significance.

The average gain of fat in the rats which received the 2% fat diet was 21.04 gm., while their food contained only 13.22 gm. Fat synthesis, therefore, took place from the diet containing the minimum amount of fat.

TABLE 4

Average amounts of food eaten during 70 days; and initial and final nitrogen and fat contents of rat bodies.[1]

FAT CONTENT OF DIETS	FOOD EATEN	INTAKE OF NITROGEN	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	gm.	gm.	gm.	gm.	gm.	gm.
2	703.4	22.507	1.17	6.86	3.72	24.75
5	672.9	22.507	1.16	7.24	3.69	26.96
10	627.6	22.507	1.14	7.50	3.62	27.79
30	494.4	22.507	1.15	7.59	3.66	27.73

[1] The composition of the bodies of the rats at the beginning of the experiment was computed from the directly determined composition of a control group; the composition of the rat bodies at the end of the experiment was determined by chemical analysis.

The per cent of recovery of feed nitrogen in the nitrogen of the urine, feces and body gain for the rats which received the 2, 5, 10 and 30% fat diets were 98, 98, 99 and 99, respectively.

The data in table 5 show that there were slight decreases in the nitrogen of the urine, much larger decreases in the nitrogen of the feces, and increases in the nitrogen retained, during the 70-day experiment, in the increasing order of the fat contents of the diets. The odds that the nitrogen retention from the 30% fat diet was higher than from the 2% fat diet were 253:1. Since the protein intake in all diets was the same, these observations signify that the increasing fat content of the isocaloric diets favored both the digestion and

the fundamental utilization of the protein present, the nitrogen retention from the 30% fat diet being 9.8% greater in amount than that from the 2% fat diet.

From the data in table 6 it is evident that the difference in the fat content of the diets was associated with only slight differences in the outgo of energy in feces, urine and heat, the odds that the heat production from the 30% fat diet was

TABLE 5

Partition of average nitrogen intake per rat during 70 days.

FAT CONTENT OF DIETS	NITROGEN			
	Intake	Output in		Retained
		Urine	Feces	
%	*gm.*	*gm.*	*gm.*	*gm.*
2	22.51	14.34	2.06	6.11
5	22.51	13.95	2.03	6.53
10	22.51	14.10	1.86	6.55
30	22.51	14.10	1.70	6.71

TABLE 6

Partition of average daily intake of food energy per rat during 70 days.

FAT CONTENT OF DIET	GROSS ENERGY INTAKE	ENERGY INTAKE				ENERGY OUTPUT			ENERGY RETAINED
		Protein	Carbohydrate	Fat	Metabolizable	In feces	In urine	As heat	
%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
2	2854	799	1912	143	2601	128	125	2195	406
5	2854	799	1713	342	2605	126	123	2165	440
10	2854	799	1456	599	2610	121	123	2154	456
30	2854	799	657	1398	2615	123	116	2155	460

less than from the 2% fat diet being 59 to 1; but the differences in energy retained were much more significant, the odds being 253 to 1 that the amount of energy retained from the 30% fat diet was greater than that retained from the 2% fat diet. It is a coincidence, and not an error, that the odds, as stated, relating to nitrogen and energy retention were in both cases 253:1. Of the difference in the amounts of energy stored from the 2% and the 30% fat diets 54.6% was from protein.

Since the subjects of this experiment were allowed normal freedom of exercise, it was not determined whether differences in the amount of voluntary exercise taken by these growing rats on the isocaloric diets of different fat content contributed to the observed differences in heat production and in energy retention. Smith and Conger ('44), however, found that 56% of food calories coming from fat caused no disturbance in voluntary activity, and that as much as 72% in fat calories caused only slight depression of activity. In the 30% fat diet which was fed in the present investigation the fat supplied about 50% of the energy. Further evidence on this aspect of the problem was obtained from the experiment with mature rats as subjects, with voluntary activity excluded.

With respect to the utilization of both nitrogen and energy the increased efficiency of the 5% fat diet as compared with the 2% fat diet was much greater than that of the 30% fat diet as compared with the 5% fat diet.

SUMMARY

A 70-day metabolism and body analysis experiment was conducted to determine the effects of differences in the fat content of isocaloric diets on the utilization of food energy and protein.

The subjects were four groups of 10, growing male albino rats, each of these four groups containing one rat from each of the same ten litters.

A comparison was made of four diets containing 2, 5, 10 and 30% of fat, respectively, these diets being so compounded and fed as to supply to each rat of a litter-four the same quantities of gross energy, protein, and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, with a single value of the heat production for the 70 days as the energy of the food minus the energy of the excreta and of the body gain.

The gains in live weight, the digestibility of nitrogen, and the retention of nitrogen and energy were in the order of the increasing fat content of the diets; the superiority of the 5%

over the 2% fat diet with respect to the utilization of both protein and energy being much greater than the superiority of the 30% as compared with the 5% diet.

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RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

II. BY THE MATURE ALBINO RAT [1]

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In a recent investigation of the associative dynamic effects of protein, carbohydrate and fat, by Forbes and Swift ('44), an effort was made to differentiate between the individual influences of these nutrients on the heat production by determining their specific dynamic effects separately and also in the four possible combinations of these three kinds of nutriment, each such supplement being superimposed upon a complete diet sufficient for maintenance. In thus recognizing the heat production of energy equilibrium as the standard base from which to measure nutritive energy value, it is not implied, and it is not the understanding of the authors that the heat increment of nutrients retained as body increase is the same as that of nutrients catabolized; but it is their belief that after the diet has satisfied the unavoidable nonproductive energy expense of maintenance, the heat production of energy equilibrium is the logical base value from which to measure the expense of utilization of food energy for whatever productive purposes it serves in the metabolic ensemble imposed by the experimental regime.

In the study just mentioned the two nutrient combinations which led to the minimum heat increments were those composed of beef protein and lard, and of cerelose (corn sugar)

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and lard. Next in the increasing order of dynamic effects was the combination of beef protein, cerelose and lard; while the least efficient combination, as indicated by the maximum heat increment, was the one containing no fat, and which was composed of cerelose and beef protein. The implication, therefore, was that lard, in its combinations with cerelose and with beef protein, conferred economy of utilization of food energy, as indicated by low heat increments.

As a means of throwing further light on the influence of fat on economy of food utilization, therefore, two studies, one with growing and the other with mature rats as subjects were conducted; and the first of these experiments having been discussed (Forbes, Swift, Elliott and James, '46), an account of the second is now presented.

Since the general method of this experiment involves the determination of the specific dynamic effects of nutrients, the results of which are much affected by the details of the procedure followed, the experimental requirements of this estimation will be considered. The general principle of measurement of the dynamic effects of nutriment is to determine the heat increment resulting from the feeding of the test substance to the experimental subject in some basal status. In this relation one's first thought is inevitably of Rubner's ('02) measurement of the specific dynamic effect of protein, based upon the heat production of a fast.

It is the opinion of the authors that the specific dynamic effects of nutrients, in the sense in which Rubner used the term, are without definite significance as measures of the energy expense of nutrient utilization, for the reasons that the heat production of a fast, at the expense of body nutrients, comprises two factors, a net energy and an energy expense of utilization, exactly as does metabolizable energy of food origin; and that a part of the energy value of nutrients fed to a fasting animal is inevitably used to spare the catabolism of body nutrients. Therefore the observed increase in heat production is less than the true energy expense of utilization of the food substance.

This idea of an energy expense of utilization of body nutrients catabolized is not new; in fact it was clearly expressed in connection with one experiment by Rubner ('02, p. 371) himself. In this instance he definitely interpreted the increased heat production in phlorhizin glycosuria, in comparison with that of fast, as representing a specific dynamic effect of the body protein catabolized.

Without undertaking to review all the literature on this point, a few of the many supporting expressions may be cited. Adams's ('26) confirmation of Oppenheimer's theory that the cells of the body must do chemical work in order to bring about some of the metabolic functions following the ingestion of food, by reference to the second and third laws of thermodynamics. Another is the proposal by Williams, Riche and Lusk ('12) of a method of calculating specific dynamic effects of protein which recognizes the dynamic effect of body protein catabolized during fast. Lusk ('28) positively expressed his approval of Rubner's conclusion that there is a specific dynamic effect of catabolized body protein. Borsook and Winegarden ('31) observed a close correlation between the specific dynamic effect of protein and the increase in urinary nitrogen excretion as compared with that of the basal levels. Finally, we may cite Borsook's ('36) conclusion that there is no reason to expect different energy exchanges whether tissue protein and amino acids, or amino acids immediately derived from ingested proteins, are metabolized. The subject of dynamic effects of body nutrients has also been discussed by Forbes and Swift ('41), who presented results of an effort to measure such values.

In spite of Rubner's ('02, p. 371) conclusion that there is a dynamic effect of body nutrients catabolized, and the general approval of the principle involved, however, this conception has not been generally followed through to its logical consequence in the determination of dynamic effects of nutrients, either by Rubner or by those who have followed his lead. This logical consequence would be the recognition of the fasting catabolism minus the dynamic effect of the body nutrients

catabolized as the theoretically correct base value of heat production. An excuse, if not an adequate reason, for the general failure to recognize this point of view is that there seems to be no entirely defensible method for determining the dynamic effect of body nutrients catabolized.

It is, therefore, the understanding of the authors that determinations of dynamic effects of nutrients made with any submaintenance base value of heat production are in error, as observed, by the amount by which the metabolizable energy of the test nutrients serves to diminish the dynamic effect of the body nutrients catabolized.

In addition to this error, other reasons why the heat production at a submaintenance plane of nutrition is not a favorable one from which to measure the dynamic effects or heat increments of nutrients are (a) that nutrition at a submaintenance level is quite irksome to the experimental subject, and is therefore likely to lead to inconsistent response to the food; and (b) that the heat production at a submaintenance plane of nutrition is a difficultly measurable value, without standard significance, since its magnitude is decreased during the continuance of the submaintenance status by a rapid decrease in heat from carbohydrate, a slower decrease in heat from protein, and a decrease in heat from fat the extent of which is largely dependent on the fatness of the animal.

According to the authors' understanding the determination of the most significant measurements of the energy expense of nutrient utilization depends upon having the experimental conditions such that there are the minimum differences in the quantities of nutrients of the kinds metabolized in the two measurements of heat production compared. These conditions seem to be met in the most practicable manner when the test nutrient is superimposed upon a nutritively complete diet fed in a quantity sufficient for energy equilibrium.

Another extremely important consideration relating to the determination of the energy expense of utilization of nutrients is the method of feeding — whether the effect as observed is a short-time measurement derived from a single portion

of the test substance fed to an animal without preliminary treatment, or from a much longer-time measurement at an established plane of nutrition to which the animal has been adapted by adequate preparatory treatment.

Three reasons why heat increments should be determined by difference between results from established states of nutrition rather than from single feedings of test nutrients are the following: (a) animals are highly adaptable to conditions of nutrition, and the economy with which they utilize nutrients is much affected by the need for thrift; a heat increment representing conditions to which an animal has become adjusted, therefore, is much more reliably representative of the diet than is an increment representing a single feeding to an animal not so adjusted, (b) periods of heat measurement with animals which have been adjusted to their diet by preliminary treatment may be as long as required in the interest of accuracy, while observations based on single feedings are limited to intervals between times of feeding, (c) in the single-feeding method an accurate measurement of the heat increment is rendered impracticable by the technical difficulty of accounting for all of the increase in heat; in fact this frequently is not even attempted, conclusions being based on the highest heat production observed, or on the rate of heat production during some short time interval, the values so derived in either case standing in no particular relation to the total heat increment.

It is also favorable to consistency of heat increments that the experimental subjects be habituated to the other environmental conditions, especially as to air temperature, which are to prevail during the determination.

Furthermore, the experience of the authors suggests that there are seasonal effects to be reckoned with in research in energy metabolism, and therefore a program of experimentation running through a number of months should be so planned that the accumulation of a satisfactory volume of evidence is accomplished not by a single, inclusive experiment requiring the whole period for its execution, but by repeated ex-

periments with smaller numbers of subjects at a time, each such sub-experiment being complete in the sense of covering the entire ground of the project.

In the determination of heat increments based on the heat production of approximate energy equilibrium it is assumed that the energy expense of maintenance is unaffected by the plane of nutrition, and that this quota cancels out in the computation of the increments, the difference in heat production at the two planes of nutrition, therefore, representing the energy expense of food utilization.

EXPERIMENTAL

The purpose of the present experiment was to determine the energy expense of utilization (dynamic effect) of complete diets as affected by their fat contents. The experiment was conducted by the open-circuit, respiratory quotient method of Haldane, with the mature albino rat as the subject. The diets contained 2, 5, 10 and 30% of fat, respectively, and were so compounded and fed as to supply identical quantities of energy, protein, and vitamins. The composition of the diets fed in this experiment was exactly the same as that of the diets fed in the experiment reported in the preceding paper (see its table 1), except that during the last quarter of the experiment the intake of calcium pantothenate was increased five-fold, to 250 mg. per kilogram of diet no. 1, and of isocaloric quantities of the other diets.

The daily intake of gross nutrients is recorded in table 1, in which it is shown that while on either maintenance or supermaintenance feeding the mature rats on the four diets received the same quantities of energy and of protein, but different quantities of fat, and compensating differences in carbohydrate.

In table 2 is given the general schedule of experimentation. The subjects were 48 male rats constituting four groups of 12 each, one group for each diet, each of these four groups containing one rat from each of 12 litters. The rats had been reared for the experimental program at temperatures within

the zone of thermal neutrality, and were born on such dates that each individual was 205 days old when first used in the respiration chamber on the maintenance diet, and 212 days old when first used on the supermaintenance diet.

The heat production was measured for each rat for each dietary treatment during the greater part of 2 consecutive working days; heat increments being determined as the difference in heat production of the same 12 rats on maintenance

TABLE 1

Percentage contents of diets, and daily intake of protein, carbohydrate, fat and energy.

DIET NO.	PLANE OF NUTRITION	TOTAL FOOD INTAKE	PROTEIN		CARBOHYDRATE		FAT		ENERGY
		gm.	%	*gm.*	%	*gm.*	%	*gm.*	*Cal.*
1	Maintenance	11.00	20.00	2.20	67.78	7.46	2.07	.23	44.64
2	Maintenance	10.52	20.90	2.20	63.68	6.70	5.07	.53	44.64
3	Maintenance	9.81	22.41	2.20	57.66	5.66	10.08	.99	44.64
4	Maintenance	7.73	28.48	2.20	32.70	2.53	30.10	2.33	44.64
1	Supermaintenance	16.00	20.00	3.20	67.78	10.84	2.07	.33	64.93
2	Supermaintenance	15.31	20.90	3.20	63.68	9.75	5.07	.78	64.93
3	Supermaintenance	14.28	22.41	3.20	57.66	8.23	10.08	1.44	64.93
4	Supermaintenance	11.25	28.48	3.20	32.70	3.68	30.10	3.39	64.93

TABLE 2

Schedule of experimentation.

RAT NOS.	PLANES OF NUTRITION	DAILY ENERGY INTAKE	AGE OF RATS	AVE. WEIGHTS OF RATS	DATES OF RESPIRATION MEASUREMENTS
		Cal.	*days*	*gm.*	
1–12	Maintenance	44.64	205	353	Jan. 29–Feb. 3
1–12	Supermaintenance	64.93	212	381	Feb. 5–10
13–24	Maintenance	44.64	205	314	Feb. 26–March 3
13–24	Supermaintenance	64.93	212	336	March 5–10
25–36	Maintenance	44.64	205	319	April 23–28
25–36	Supermaintenance	64.93	212	344	April 30–May 5
37–48	Maintenance	44.64	205	320	May 21–26
37–48	Supermaintenance	64.93	212	344	May 28–June 2

and supermaintenance quantities of the same diet. Voluntary activity during respiration experiments was excluded by subjecting the rats to a bright light, which made them keep their eyes shut and remain generally motionless.

In order to equalize possible effects of the time of year on the results, the investigation was conducted as a sequence of four identical programs each of which covered the whole ground of the study, with three rats as subjects. Therefore twelve rats in all were used with each experimental treatment.

The subjects were selected on the basis of their performance during 5 days' ad libitum consumption of the colony diet in individual cages. Then the selected individuals were fed a constant amount (16 gm.) of the colony diet, twice daily, for 5 days, to establish them, as nearly as possible, on a uniform rate of metabolism throughout the day, and to determine that they would probably eat equivalent quantities of the experimental diets when later called upon to do so. The subjects were then fed maintenance quantities of the four experimental diets for 10 days, to adapt them to this plane of metabolism; and during this interval each rat was subjected to the routine of the respiration experiment, on 2 days, by way of training for the respiration measurements to follow.

This preliminary maintenance period was followed by 8 days' feeding on the same quantities of the diets, with collection of the excreta for the determination of nitrogen balance and metabolizable energy. Then followed 2 days of respiration measurement on the maintenance diets. The subjects were next given the supermaintenance diets for 5 days to adapt their heat production to this level of food intake, the weight of the diets being 16 gm. for diet no. 1, and somewhat less for the diets of higher fat contents as required to provide isocaloric food intake. Next followed 2 days of respiration measurement, and finally an 8-day period of excreta collection, both on the supermaintenance diets.

A purpose in the order of feeding treatment, as recounted, was to have the respiration experiments on the two planes of food intake as close together as possible, that is, separated

only by the 5 days required to establish the animals in heat production representative of the higher plane of food consumption.

During the 2 days' measurements of the heat production, the rat, after receiving food at 6:30 A.M., was put into the respiration apparatus at 8:00 A.M., where it remained until 3:00 P.M.

The weight of the carbon dioxide absorbed, and the degree of physical activity of the experimental subject as indicated by a work adder, were recorded each hour from 9:00 A.M. until 3:00 P.M., and the hourly values used for heat production were selected as those which did not differ from the average for the day (excluding the first hour) by more than 6%. Incidentally this exclusion of the more aberrant data made no significant difference in the experimental findings. The weight of carbon dioxide eliminated during the entire 7-hour period was used in computing the respiratory quotient, but only the amount eliminated during the selected intervals of quiet were used in computing the heat production. It was assumed that the total respiratory quotient was unaffected by the slight activity of the animal during the 7-hour respiration period. The definite significance of the results obtained, therefore, was limited to the selected intervals of quiet, without evidence or claim that they exactly represented the entire 24-hour day. It is also conceded that there was probably a difference between the amounts of heat produced by the subjects while asleep and while awake but motionless.

The respiration experiments were conducted with the apparatus at a temperature of 28°C. $\pm$ 0.1°C., the temperature inside the respiration chamber being about 29°C., which is within the zone of thermal neutrality. When the rats were not in the respiration chambers they were in a room the temperature of which was thermostatically controlled at 29°C.

The data in table 3 show that the nitrogen of the urine during the intended maintenance feeding was close to the requirement for equilibrium under the prevailing dietary conditions; and the greater amount of nitrogen in the urine

from supermaintenance than from maintenance feeding may be considered as resulting from the materially greater amount of digested nitrogen available. While it would be desirable, in determining dynamic effects of nutrients, to have the same quantity of nitrogen catabolized in both the basal and the supplemented periods, this is apparently an impossible objective. On comparing the nitrogen retention from the 2% and the 30% fat diets by these approximately mature rats it appears that the odds were only 4 to 1 that the observed

TABLE 3

Utilization of daily nitrogen.

DIET NOS.	PLANE OF NUTRITION	FAT CONTENT OF DIET	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION
		%	*mg.*	*mg.*	*% of intake*	*mg.*	*% of intake*	*% of intake*
1	Maintenance	2	352	319	90.6	325	92.3	— 1.7
2	Maintenance	5	352	318	90.3	327	92.9	— 2.6
3	Maintenance	10	352	320	90.9	316	89.8	1.1
4	Maintenance	30	352	322	91.5	330	93.8	— 2.3
1	Supermaintenance	2	512	461	90.0	415	81.1	9.0
2	Supermaintenance	5	512	461	90.0	416	81.3	9.0
3	Supermaintenance	10	512	463	90.4	415	81.1	9.4
4	Supermaintenance	30	512	467	91.2	410	80.1	11.1

difference was significant; the odds were many millions to one that the corresponding values for energy retention were significant.

The metabolizable energy values of the daily food (table 4) were virtually constant for the four diets at the maintenance and at the supermaintenance planes of food intake, with a range of coefficients of variation from 0.3% to 0.8%.

The heat production from the daily food intake, however, at both planes of nutrition, diminished in the increasing order of the fat contents of the diets, without exception; and the coefficients of variation of the heat production (3.0% to 5.6%), while much higher than for metabolizable energy, were satisfactorily low for this observation.

The average coefficient of variation of the determined values for metabolizable energy of the four diets was 0.46%, and for the heat production from these diets it was 4.1%; and the computed odds that the average heat increment value of the 30% fat diet was less than that of the 2% fat diet were many millions to one. Of the greater amount of energy stored 67% was protein.

With respect to the plane of nutrition of the rats during the period of approximate energy equilibrium — three groups lost and one group gained slight amounts of nitrogen (table 3); three groups gained slightly in weight, while the fourth exactly maintained its weight; and the values for heat production subtracted from those for metabolizable energy (table 4) show that all groups stored energy during the respiration experiments. Conditions during the maintenance period, therefore, are considered to have been satisfactory for the determination of the base value of heat production.

TABLE 4

Partition of daily food energy.

DIET NOS.	PLANES OF NUTRITION	INTAKE	FECES	URINE	METABOLIZABLE ENERGY		HEAT PRODUCTION	
		Cal.	*Cal.*	*Cal.*	*Cal.*	*coef. var. %*	*Cal.*	*coef. var. %*
1	Maintenance	44.64	2.25	2.75	39.64	.3	30.50	5.6
2	Maintenance	44.64	2.35	2.75	39.54	.4	29.93	3.8
3	Maintenance	44.64	2.16	2.75	39.73	.5	29.50	3.2
4	Maintenance	44.64	2.53	2.76	39.35	.8	27.56	5.4
1	Supermaintenance	64.93	3.30	3.91	57.72	.3	37.79	3.8
2	Supermaintenance	64.93	3.27	3.92	57.74	.4	36.32	3.2
3	Supermaintenance	64.93	3.20	3.93	57.80	.5	35.28	3.0
4	Supermaintenance	64.93	3.58	3.96	57.39	.5	31.66	4.8

The gross energy of the daily dietary supplements was 20.29 cal.; and the daily heat increments (table 5), which diminished in the increasing order of the fat contents of the diets, were 7.29, 6.39, 5.78 and 4.10 cal., respectively, which amounted to 36, 31, 29 and 20%, respectively, of the gross energy of these supplements containing 2, 5, 10 and 30% of fat.

A logical question, however, as to the validity of this apparent effect of the fat content of the diet on the efficiency of utilization of food energy concerns the extent to which the requirements of energy production from fat and from carbohydrate, especially the vitamin requirements, were satisfied by the diets. It was intended that the vitamin intake should be liberal, and the amounts of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate and

TABLE 5

Quantities, increments and sources of average daily heat production.

DIET NOS.	PLANES OF NUTRITION	TOTAL HEAT, AND HEAT INCREMENTS	NON-PROTEIN R. Q.	SOURCES OF HEAT PRODUCTION			
				Protein	Carbohydrate	Fat	Fat synthesis
		Cal.		*Cal.*	*Cal.*	*Cal.*	*Cal.*
1	Supermaintenance	37.79	1.18	11.01	25.72	0	1.06
1	Maintenance	30.50	1.09	8.63	21.47	0	0.40
1	Heat increments	7.29		2.38	4.25	0	0.66
2	Supermaintenance	36.32	1.15	11.03	24.50	0	0.79
2	Maintenance	29.93	1.05	8.68	21.02	0	0.23
2	Heat increments	6.39		2.35	3.48	0	0.56
3	Supermaintenance	35.28	1.07	11.00	23.91	0	0.37
3	Maintenance	29.50	1.00	8.36	21.10	0	0.04
3	Heat increments	5.78		2.64	2.81	0	0.33
4	Supermaintenance	31.66	0.85	10.87	10.54	10.25	0
4	Maintenance	27.56	0.82	8.76	7.57	11.23	0
4	Heat increments	4.10		2.11	2.97	— 0.98	0

niacin in the diets were the same as those characterized by Boutwell and associates ('45) as being at a high level. In the course of this experiment the authors received a suggestion to the effect that an increased pantothenic acid intake might wipe out the appearance of superior economy of utilization of fat as compared with carbohydrate.

In response to this suggestion an increase was made in the calcium pantothenate intake, and during the last quarter of

this experimental program, five times as much of this vitamin was fed as during the first three-fourths of the program. This increased intake of calcium pantothenate, however, did not alter the general trend of the results. The individual variation of the heat production of the rats, and apparent effects of the season of the year, were of such magnitude that the comparison of the results from the last three rats with the results from the previous nine afforded no significant basis for a conclusion as to the pantothenic acid requirement.

The observed results with respect to the effect of the fat content of the diets on the economy of utilization of food energy, therefore, may be considered as being in accord with the present state of knowledge of energy metabolism; but it is necessary to concede the possibility that future progress in understanding of the nutritive requirements of energy production from fat and carbohydrate could alter these findings.

In relation to the origin of the heat production, with the increasing fat and compensating decrease in the carbohydrate content of the diets, the amount of heat derived from protein was virtually unchanged; the heat of carbohydrate catabolism was decreased; there was no heat from fat catabolism except from the 30% fat diet; and there was a decreasing quantity of heat from fat synthesis, there being no heat from this source from the 30% fat diet. The sources of the decreasing energy expense of utilization of the isocaloric diets, in the order of their increasing fat contents, therefore, were decreasing heat from the catabolism of carbohydrate and from fat synthesis. In this factoring of the heat increment, the portion ascribed to fat synthesis was conventionally considered to be exclusively a product of carbohydrate catabolism, whereas, it will be understood, protein also presumably contributed to this quota.

The heat increments reported from this investigation were of a general order of magnitude similar to those derived in other recent experimental work conducted by the same procedure in this laboratory. The reason that they were much larger than those commonly reported in the literature lies

primarily in the difference in the base values from which the increments were computed. In the present study these base values of heat production were near to that of energy equilibrium, the increase in heat production incident to the consumption of the test supplements, therefore, representing the increased energy expense of food utilization; while most of the determinations of dynamic effects reported in the literature have been determined by procedures implying some submaintenance level of heat production as the base value, this value being too high, and the heat increments too low, to represent the food correctly, the extent of this error being that to which the heat increment as observed had been diminished by heat derived from body nutrients catabolized.

For the avoidance of confusion, the authors call attention to the facts that the results reported in the present and the foregoing paper represent growing animals during voluntary activity, and mature animals at rest, on diets comparatively low in fat content, by virtue of which they are not in conflict with the numerous published findings that work is performed more efficiently at the expense of carbohydrate than of fat.

SUMMARY

Respiration experiments were conducted by the open-circuit, Haldane respiratory quotient procedure, with 48 mature albino rats as subjects, to investigate the energy expense of utilization (heat increment) of complete diets as affected by their contents of fat.

Heat increments were measured as the difference in heat production from maintenance and supermaintenance diets containing 2, 5, 10 and 30% of fat, respectively, fed in such a way as to supply equal quantities of gross energy, of protein, and of vitamins.

In harmony with results of a growth experiment in which the same diets were fed, the digestibility and the retention of food nitrogen were highest when the diet containing 30% of fat was used.

The metabolizable energy of the diets was unaffected by their fat contents.

The heat production at both planes of nutrition, and also the heat increments, diminished in the order of the increasing fat contents of the diets.

The heat increments of the dietary supplements containing 2, 5, 10 and 30% of fat, respectively, were equivalent to 36, 31, 29 and 20%, respectively, of their gross energy.

The decreasing energy expense of utilization of the isocaloric diets, in the order of their increasing fat contents, was due to decreasing heat from the catabolism of carbohydrate and from fat synthesis.

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VITAMIN A, ASCORBIC ACID AND SPINAL FLUID PRESSURE RELATIONSHIPS IN THE YOUNG BOVINE [1]

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In a previous publication Moore and Sykes ('40) reported that vitamin A deficiency in the young bovine was associated with an increased spinal fluid pressure. In extreme vitamin A deficiency pressures as high as 600 mm. of saline were noted by Moore and Sykes ('41), an increase of about six-fold above normal. Boyer, Phillips and co-workers ('42) suggested that the increased spinal fluid pressure noted in vitamin A deficiency was associated with a decreased synthesis of ascorbic acid. These workers produced a reduction in spinal fluid pressure by the subcutaneous injection of ascorbic acid in three out of five calves. Moore, Berry and Sykes ('43) found no particular relationship between the level of blood plasma ascorbic acid and increased spinal fluid pressure. However, the recorded pressures were not high and the variations in carotene intake were small so that the authors withheld further comment until more critical experiments were completed. These experiments have been completed and are reported herein.

EXPERIMENTAL

Three groups of calves were used. The three calves in the first group had been receiving various quantities of carotene

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in the form of dehydrated alfalfa leaf meal in studies on carotent requirements. Calf 468 had been receiving sufficient carotene to maintain a normal spinal fluid pressure. Calves 474 and 472 had not been receiving sufficient carotene to maintain a normal spinal fluid pressure and thus showed some elevation of pressure at the time they were used in the present experiment. The carotene supplement was removed from the ration of all three calves in order to cause increases in spinal fluid pressure. One of these calves, animal 468, after developing some increased spinal fluid pressure was then injected subcutaneously with 1 gm. of ascorbic acid daily for 13 days.

The two calves used in group II were similar to those in group I. These two calves had been receiving sufficient carotene to prevent any increase in spinal fluid pressure. The carotene supplement was removed from the ration at which time 3 gm. of chlorobutanol per day were added to the ration. Since several laboratories have shown that this drug stimulates the synthesis of ascorbic acid in animals, the purpose in this instance was to determine whether or not the extra synthesized ascorbic acid would prevent the development of an increased spinal fluid pressure in the vitamin A deficient calf.

There were two calves in group III. They were about 3 months of age and had not been used previously for experimental purposes. They were placed on the vitamin A deficient ration with adequate supplementation for 30 days after which the supplement was cut down to variable amounts. The supplement was fed in just sufficient quantity to maintain fair health and appetite, but not in sufficient quantity to prevent an increase in spinal fluid pressure. Begining at 183 days of age 1 gm. of ascorbic acid per day was injected subcutaneously. The purpose of using these calves was to maintain normal or above normal ascorbic acid values in blood and spinal fluid and to determine whether or not the vitamin A deficient calf would still develop an increased spinal fluid pressure.

Plasma vitamin A and carotene determinations were made each week by a modification of a previously published pro-

cedure for carotene by Moore ('39). Plasma ascorbic acid determinations were made by the method of Mindlin and Butler ('38). When spinal punctures were made 3 ml. of spinal fluid were withdrawn on which ascorbic determinations were made by the same procedure as used for blood plasma. Where ascorbic acid determinations were made on whole blood, the method of Roe and Keuther ('43) was used. A photoelectric spectrophometer was used for the chemical determinations.

Spinal punctures were made by the method of Sykes and Moore ('42). The pressure was measured by connecting the puncture needle to a small bore glass tube. The height to which the spinal fluid rose in the tube plus the length of the needle was recorded as the spinal fluid pressure.

When 1 gm. of ascorbic acid was injected subcutaneously, just prior to use it was dissolved in 15 ml. of water containing 750 mg. of trisodium phosphate, which partially neutralized the ascorbic acid.

The blood and spinal fluid samples used for chemical analysis were drawn about 18 hours after the previous subcutaneous injection of ascorbic acid.

RESULTS AND DISCUSSION

The results with the first group of three calves are shown in table 1 which confirms in part the observations made by Boyer and coworkers ('42). Thus when the carotene supplement was removed from the ration of animal 468 there was an increase in spinal fluid pressure associated with a depression in plasma vitamin A, carotene, ascorbic acid and spinal fluid ascorbic acid values. The plasma ascorbic acid values were not depressed until after 441 days. However, the injection of 1 gm. of ascorbic acid for a period of 13 days caused no depression in spinal fluid pressure.

Practically all the plasma ascorbic values shown in table 1 for animals 472 and 474 were low even before the removal of the alfalfa meal. These two animals had not been receiving sufficient carotene to maintain a normal spinal fluid pressure so that the low plasma values even before the removal of the

TABLE 1

Effect of vitamin A deficiency on spinal fluid pressure, plasma and spinal fluid ascorbic acid values.

AGE	PLASMA			SPINAL FLUID	
	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure
days	*μg./100 ml.*		*mg./100 ml.*		*mm. H_2O*
			Animal 468		
357	6.9	38	0.33	...	..
371	10.8	33	0.36	3.72	95
392	9.3	29	0.30	...	...
406	9.3	37	0.37	...	...
413	8.4	39	...	3.44	100
alfalfa meal removed at 417 days					
420	6.3	33	0.43	...	...
427	4.5	23	0.46	...	...
434	3.3	17	0.38	..	...
441	2.7	12	0.27	1.02	150
448	0.9	12	0.20	...	...
455	1.6	10	...	1.86	240
462	1.2	8	0.27	...	...
1 gm. ascorbic acid daily at 463 days					
469	0.3	10	0.32	...	...
476	..	..	0.25	1.42	280
			Animal 472		
363	6.0	53	0.13	...	160
377	10.5	40	...	...	..
384	10.5	47	0.14	...	...
398	9.6	29	0.20		
405	10.8	40	...	2.18	180
alfalfa meal removed at 409 days					
412	9.0	32	0.28	...	...
419	4.8	25	0.21	...	...
426	1.8	10	0.30	...	...
433	0.9	6	0.26	2.42	270
440	0.9	4	0.21	...	...
447	1.8	7	0.22	2.01	210
454	3.1	5	0.23	...	...
461	1.8	7	0.15	...	
468	..	..	...	0.66	155
			Animal 474		
359	7.5	25	0.11	3.07	170
380	7.2	32	0.26	...	...
387	7.1	25	...	...	...
394	9.0	34	0.24	...	
401	3.9	25	..	2.67	170
alfalfa meal removed at 405 days					
408	6.3	22	0.33	...	...
415	2.7	18	0.33	...	...
439	0.3	9	0.28	1.59	300
446	0.9	5	0.23	...	
453	1.8	3	0.25	1.49	310
460	..	3	0.22	...	...
467	0.3	4	0.23	...	
474	1.8	2	0.37	...	255

source of carotene would be expected and in line with the observations of Boyer and coworkers ('42).

The results with the second group of two calves are shown in table 2. When the source of carotene was removed from the ration 3 gm. of chlorobutanol per day were added. In both calves the plasma vitamin A and carotene values decreased when the alfalfa meal was removed from the ration. The

chlorobutanol maintained normal ascorbic acid values in both plasma and spinal fluid. Nevertheless, the spinal fluid pressure increased. When the chlorobutanol was discontinued and 1 gm. of ascorbic acid per day was injected subcutaneously for 10 days there was no decrease in spinal fluid pressure.

TABLE 2

Effect of feeding chlorobutanol on spinal fluid pressure, plasma and spinal fluid ascorbic acid values in vitamin A deficiency.

	PLASMA			SPINAL FLUID			PLASMA			SPINAL FLUID	
AGE	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure	AGE	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure
days	*µg./100 ml.*		*mg./100 ml.*		*mm. H_2O*	*days*	*µg./100 ml.*		*mg./100 ml.*		*mm. H_2O*
	Animal 466						*Animal 467*				
442	11.7	65	...	...	...	403	7.8	71	0.28	2.40	100
449	10.8	64	...	3.32	90	410	11.4	67	...	...	...
456	11.7	59	...	...	...	417	10.2	61	...	...	...
463	10.2	59	0.36	...	...	424	10.2	62	0.31	...	...
484	10.2	58	0.31	...	...	431	10.8	73	...	...	...
491	13.2	55	...	2.81	80	445	12.9	73	...	2.26	105
alfalfa meal removed at 499 days						alfalfa meal removed at 449 days					
498	10.8	68	0.49	...	...	452	12.0	59	0.34	...	...
3 gm. chlorabutanol daily at 503 days						3 gm. chlorabutanol daily at 454 days					
505	9.0	38	0.59	...	...	459	6.3	28	0.38	...	...
512	5.7	31	0.44	...	...	466	6.3	22	0.28	...	...
519	3.3	18	0.28	3.33	190	473	4.2	14	..	2.15	180
526	3.3	17	0.34	...	...	480	2.4	12	0.50	...	...
533	3.0	12	0.29	...	200	487	3.3	10	0.44	2.30	210
540	3.6	14	0.39	...	...	494	2.1	5	0.46	...	...
chlorabutanol discontinued						501	0.9	9	0.48	...	...
1 gm. ascorbic acid daily at 544 days						508	..	7	0.33	2.51	250
547	2.1	21	0.51	...	...						
554	3.0	19	0.21	...	300						

The results of injecting ascorbic acid for a long period of time into calves on subminimal quantities of carotene are shown in table 3. As a result of the low level of carotene intake the plasma vitamin A and carotene values were depressed. As a result of injecting 1 gm. of ascorbic acid per day the ascorbic acid values for plasma, whole blood and spinal

fluid were maintained at a normal level. However, the spinal fluid pressure increased.

The results obtained with the three groups of calves show that ascorbic acid is not involved in the maintenance of a normal spinal fluid pressure in vitamin A deficient calves as indicated by Boyer and co-workers ('42). When normal blood and spinal fluid ascorbic acid values were maintained by the feeding of chlorobutanol or the subcutaneous injection of ascorbic acid the spinal fluid pressure increased as the vitamin

TABLE 3

Effect of subcutaneous injection of ascorbic acid on spinal fluid pressure, plasma and spinal ascorbic acid values in vitamin A deficiency.

	PLASMA			WHOLE BLOOD	SPINAL FLUID			PLASMA			WHOLE BLOOD	SPINAL FLUID	
AGE	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Ascorbic acid	Pressure	AGE	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Ascorbic acid	Pressure
days	*μg./100 ml.*		*mg. per 100 ml.*			*mm. H_2O*	*days*	*μg./100 ml.*		*mg. per 100 ml.*			*mm. H_2O*
	Animal 88							*Animal 87*					
109	12.0	36	0.39	0.83	...	...	109	12.6	56	0.39	1.02	...	...
116	11.4	26	0.47	0.67	...	...	116	15.0	61	.	0.67	...	...
123	21.0	58	0.45	0.77	...	...	123	19.2	76	0.47	1.07	...	...
130	13.1	34	0.42	0.87	...	...	130	16.5	76	0.55	1.06	...	...
137	13.1	16	0.44	0.77	...	...	137	14.1	51	0.44	0.77	...	...
144	5.7	10	0.30	0.72	...	...	144	9.9	20	0.50	0.93	...	...
151	3.3	5	0.41	0.61	...	...	151	9.0	7	0.55	0.63	...	...
166	5.7	15	0.46	0.71	3.12	60	166	9.6	16	0.54	0.81	2.43	60
179	7.5	16	0.23	0.31	...	...	179	6.0	15	...	0.68	...	...
1 gm. ascorbic acid per day at 181 days							1 gm. ascorbic acid per day at 181 days						
186	..	..	0.53	0.85	3.45	90	186	..	..	0.44	0.79	3.18	90
200	2.1	7	0.37	0.98	...	...	200	2.1	6	0.37	1.01	...	...
207	0.9	2	0.36	0.62	3.07	120	207	2.7	4	0.61	0.95	3.34	90
214	1.2	3	0.51	0.91	...	...	214	2.4	5	0.53	1.01	...	...
221	4.2	7	0.44	0.86	...	...	221	3.9	8	0.44	0.87	...	...
228	..	5	0.45	0.78	3.32	200	228	1.5	6	0.46	1.02	...	...
235	1.2	2	0.44	0.70	...	...	235	0.6	4	0.31	0.96	3.37	160
242	3.9	7	0.47	0.83	3.24	220	242	3.9	5	0.51	0.90	...	...
249	3.6	10	0.54	0.70	...	...	249	3.9	5	0.45	0.70.	...	...
256	3.9	8	0.56	0.93	...	...	256	..	..	0.43	0.83	...	...
263	2.1	5	0.37	0.71	3.01	230	263	3.0	3	0.30	0.90	2.88	180
270	1.8	5	0.57	0.76	...	...	270	2.4	3	0.55	0.80	...	...

A deficiency progressed. Therefore, some other explanation for the mechanism of the increase in spinal fluid pressure in vitamin A deficiency in calves must be sought.

Boyer and co-workers ('42) obtained a depression of spinal fluid pressure in three out of five calves by the subcutaneous injection of ascorbic acid. However, they make one very significant statement. "Administration of ascorbic acid for periods longer than 1 week was impractical because of the moribund condition of the calves." It should be noted that Moore and Sykes ('41) state in a paper prior to the one published by Boyer and co-workers ('42), "In two cases there was a terminal drop in pressure from a previously high level. Usually the animals in this condition had very little appetite, showed diarrhea and were more or less in a moribund state so that the drop was not surprising." Apparently the Wisconsin workers injected ascorbic acid at a time when the calves naturally showed a drop in spinal fluid pressure because of their moribund condition.

Boyer, Phillips and co-workers ('42) further suggest that ascorbic acid may have some direct bearing on bone anomalies characteristic of vitamin A deficiency. It is of considerable interest that after 12 months on a very low carotene intake the two calves considered in the present report which have received daily subcutaneous injections of ascorbic acid have gone blind due to constriction of the optic nerve as reported by Moore, Huffman and Duncan ('35).

SUMMARY AND CONCLUSIONS

1. Calves rendered severely vitamin A deficient showed an increased spinal fluid pressure accompanied by depressed ascorbic acid values in blood and spinal fluid.

2. However, when normal ascorbic values in blood and spinal fluid were maintained by the feeding of chlorobutanol or the subcutaneous injection of ascorbic acid there was still an increase in spinal fluid pressure in the vitamin A deficient calves.

3. It must be concluded that the disturbance in the synthesis of ascorbic acid in the vitamin A deficient calf plays no part in the mechanism of the increased spinal fluid pressure in calves.

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THE EVALUATION OF PROTEINS IN HYPOPROTEINEMIC DOGS[1]

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THREE FIGURES

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The relation between nitrogen balance and absorbed nitrogen in normal adult dogs was described by Allison and Anderson ('45) in the following equation:

$$NB = K\ (AN) - NE_o \qquad (1)$$

where NB is nitrogen balance, AN absorbed nitrogen, and NE_o, the excretion of nitrogen on a protein-free diet. The value of K was constant in the region of negative nitrogen balance but it became a decreasing variable well on the positive side. This value of K was described as a measure of the rate of change of nitrogen balance with respect to absorbed nitrogen, an index to the utilization of a protein by the dog. If NE_o is the so-called "endogenous" nitrogen then K becomes the biological value defined by Thomas and elaborated by Mitchell and co-workers (Mitchell, '44). Possibly K can be defined best as a nitrogen balance index. Seeley ('45) obtained data in hypoproteinemic dogs which suggested that this index may be greater in depleted than in normal dogs. His work revealed also, that the amount of regeneration of plasma protein was a function of the magnitude of the nitrogen balance produced.

[1] These studies were accomplished through the support and cooperation of Sharp and Dohme, Inc., E. R. Squibb and Sons, Arlington Chemical Company, Swift and Company, and Warner Institute for Therapeutic Research.

The following work was done on hypoproteinemic dogs to determine whether equation (1) describes nitrogen balance data obtained on depleted animals and if so whether depletion affects the value of K. Further information was sought also on the correlation between the regeneration of plasma proteins and nitrogen balance.

METHODS

The dogs were depleted in proteins by feeding a protein-free diet (Allison and Anderson, '45) the process of depletion being speeded by plasmapheresis (Seeley, '45). Depletion resulted in a decrease in plasma albumin which was reflected by a decrease in total plasma protein (Chow, Allison, Cole, and Seeley, '45). The degree of depletion was estimated, therefore, by the magnitude of the decrease in total plasma protein which was calculated from nitrogen determinations using the micro Kjeldahl method. Previous studies proved that plasma proteins varied from 5.6 to 7.1, averaging 6.2 gm.% in normal dogs (Allison, Dreskin, and Morris, '41).

The nitrogen balance studies were conducted similarly to those on normal dogs (Allison and Anderson, '45). Protein-free feeding periods usually preceded and followed a period of nitrogen feeding. These periods lasted 4 days during which time the daily feces and urine samples were collected and pooled for analysis. Nitrogen was determined in the urine and in the feces by the micro Kjeldahl method.

The technique used to study the regeneration of plasma proteins was the same as that described by Seeley ('45). Briefly, it was this. The dogs were depleted by feeding the protein-free diet and by a few days of plasmapheresis until the plasma protein concentration was between 4.0 and 4.5 gm. per 100 ml. plasma and essentially constant under the experimental conditions used. The depleted animals were fed protein nitrogen for 5 days and then returned to the protein-free diet until the plasma protein concentration had returned to the former depleted level. Plasma protein in grams per cent was plotted against days and the area under the curves show-

ing the increase above the depleted concentration was taken as a measure of the amount of regeneration.

RESULTS

The data in figure 1 illustrate the relationship found between nitrogen balance and absorbed nitrogen in normal and hypoproteinemic dogs fed casein. The first line represents average data previously published (Allison and Anderson, '45) obtained on normal dogs. Each of the other curves represents data obtained on single animals which had undergone protein depletion so that the plasma proteins had been reduced to 4.6, 4.6, 4.1, and 4.1 gm. per 100 ml., respectively. The value for nitrogen balance at zero absorbed nitrogen (NE_o in equation 1) varied for each dog around a mean during the series of experiments performed to establish the curve. Thus each curve was constructed by calculating all points to the average NE_o. A straight line can be drawn which will include all these points satisfactorily. Equation (1) therefore, can be applied as a linear relationship to data obtained on hypoproteinemic as well as on normal dogs in negative or low positive nitrogen balance.

Values from these same data for plasma protein concentration, absorbed nitrogen at equilibrium, nitrogen excretion on a protein-free diet (NE_o) and the slope of the line (K in equation 1) are recorded under casein in table 1. These data prove that the amount of nitrogen necessary to maintain nitrogen equilibrium (absorbed nitrogen at equilibrium) in the depleted is less than in the normal animal. Similarly NE_o is reduced in the hypoproteinemic dog. The reduction in the amount of nitrogen necessary to maintain equilibrium can be attributed in part, therefore, to reduced protein stores as reflected by the reduction in NE_o. But the value for K (biological value or nitrogen balance index) increases from an average of 0.81 in the normal to 0.93 in the most depleted dogs. Thus, insofar as K measures retention of nitrogen, casein is utilized better in the depleted animals, another reason for the decrease in absorbed nitrogen at equilibrium.

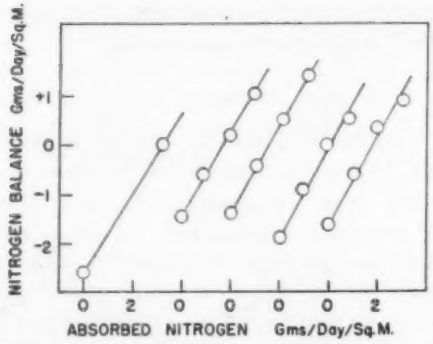

Fig. 1 Absorbed nitrogen gm./day/sq.M. plotted against nitrogen balance gm./day/sq.M. The first line represents average data obtained on normal dogs (Allison and Anderson, '45). The other four curves represent data obtained on individual hypoproteinemic dogs with plasma protein concentrations of 4.6, 4.6, 4.1 and 4.1 gm. per cent, respectively.

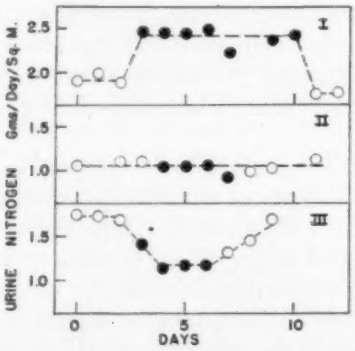

Fig. 2 Urine nitrogen gm./day/sq.M. plotted against time in days. The open circles represent data obtained while feeding protein-free diet, the solid circles data obtained while feeding protein nitrogen. See text for further description.

The following derivation illustrates the significance of K′ and "net protein" value headings for the last two columns in table 1. Absorbed nitrogen (AN) was calculated from ingested nitrogen (NI), using the so-called true digestibility (D) as follows:

$$AN = NI \times D \qquad (2)$$

Equation (1) can be rewritten therefore, as,

$$NB = (K)\ (D)\ (NI) - NE_o \qquad (3)$$

Digestibility has proven to be constant within experimental error so that,

$$NB = K'\ (NI) - NE_o \qquad (4)$$

TABLE 1

The effect of protein depletion, reflected in a decrease from normal in plasma protein concentration, upon the evaluation of proteins or hydrolysates.

DOG NO.	PLASMA PROTEIN	ABSORBED NITROGEN AT EQUILIBRIUM	NITROGEN EXCRETION ON PROTEIN-FREE DIET NE_o	K	K'	NET PROTEIN
	gm./100 ml.	*gm./day/sq.M.*	*gm./day/sq.M.*			
			Casein			
[1]	6.2	3.20	2.59	0.81	0.77	0.59
31	5.6	2.55	2.00	0.79	0.70	0.54
44	4.6	1.65	1.47	0.89	0.83	0.63
42	4.6	1.55	1.39	0.90	0.88	0.67
55	4.1	2.06	1.90	0.92	0.92	0.69
28	4.1	1.73	1.61	0.93	0.93	0.71
			Casein hydrolysate			
44	6.0	2.15	1.72	0.80	0.76	0.59
48	5.7	2.94	2.32	0.79	0.79	0.61
31	5.0	1.49	1.39	0.93	0.90	0.70
			Commercial hydrolysate B			
44	6.0	2.15	1.72	0.80	0.76	0.59
48	5.2	2.72	2.28	0.84	0.84	0.65
31	4.8	1.90	1.77	0.93	0.93	0.72
			Commercial hydrolysate C			
60	6.0	4.60	2.20	0.48	0.40	0.18
48	5.0	3.50	2.10	0.60	0.52	0.23
55	3.9	2.42	1.69	0.70	0.60	0.26
			Protein A			
[1]	6.2	6.60	2.50	0.39	0.32	0.28
50	4.4	2.90	1.65	0.57	0.51	0.45
42	4.0	1.50	1.10	0.73	0.66	0.58

[1] Data previously published (Allison and Anderson, '45).

where K′ is the rate of change of nitrogen balance with respect to NI and is proportional to K in equation (1) through digestibility. K′ increases even as does K with a tendency for the former value to approach K in the depleted animal suggesting that the factors involved in the calculation of digestibility are also altered by depletion. By multiplying K′ by the fraction of protein in the source of dietary nitrogen, the "net protein" value is obtained. This value proposed by Mitchell ('44) is a measure of the efficiency of the source to furnish protein nitrogen to the animal.

Data similar to those obtained for casein are recorded in table 1 for a casein hydrolysate,[2] for commercial hydrolysates B [3] and C,[4] and for a protein A derived from soybean. These data support the conclusions drawn from the casein data that the amount of nitrogen necessary to maintain the depleted dog is less than in the normal dog, a change which is reflected in a tendency for NE_o to be reduced and for K or K′ in equations (1) and (4), respectively, to increase. These data prove also that casein, the casein hydrolysate and commercial hydrolysate B are identical in value as measured by K or K′ (values which may be called nitrogen balance indexes of absorbed and of total dietary nitrogen, respectively).

The data in table 2 on dried egg white [5] (uncooked) have been separated from those in table 1 and given in somewhat more detail to emphasize results not observed with other proteins. The data on egg white fed to hypoproteinemic dogs are similar to the others in that absorbed nitrogen at equilibrium and NE_o are decreased while K and K′ and "net protein" value are increased above control values. There are however, outstanding differences. The value of K (0.96) which is higher than values obtained for the other proteins in normal dogs becomes greater than unity in the depleted animals. The data

[2] The casein hydrolysate was supplied by Dr. Bacon F. Chow of the Squibb Institute for Medical Research.

[3] Amigen (batch 10067) furnished by Dr. Warner M. Cox, Jr., of Mead Johnson and Company.

[4] Hydrolysate C was a commercially prepared hydrolysate.

[5] Manufactured by Swift and Company. This dried egg white is uncooked.

on dogs 54, 50, and 31 in table 2 are arranged chronologically, each determination of NE_o and NB requiring about 8 days, so that the entire experiment on each dog lasted approximately 1 month. These data prove that values for K can be greater than unity in the depleted dog. Furthermore, continued feeding of egg white results in a reduction in the value of NE_o from approximately 1.8 to 1.45 gm./day/sq.M. accompanied by a drop in K from values greater than one to approximately unity.

TABLE 2

The effect of protein depletion, reflected in a decrease from normal in plasma protein concentration, upon the evaluation of egg white.[1]

DOG NO.	PLASMA PROTEIN	ABSORBED NITROGEN AN	NITROGEN BALANCE NB	NITROGEN EXCRETION ON PROTEIN-FREE DIET NE_o	K	K'	NET PROTEIN
	gm./100 ml.	*gm./day/sq.M.*	*gm./day/sq.M.*	*gm./day/sq.M.*			
[2]	6.0	2.50	0	2.55	0.96	0.91	0.63
54	5.4	1.87	+ 0.19	1.81	1.07	1.05	0.76
	5.0	0.89	— 0.48	1.63	1.29	1.25	0.91
	5.0	1.44	+ 0.01	1.47	1.03	1.03	0.75
50	4.7	1.12	— 0.56	1.91	1.20	1.16	0.84
	4.9	0.84	— 0.58	1.49	1.08	1.08	0.78
	4.9	1.44	— 0.04	1.45	0.99	0.99	0.72
31	4.9	1.63	+ 0.30	1.77	1.27	1.27	0.92
	4.7	0.85	— 0.37	1.79	1.67	1.67	1.19
	4.7	1.26	— 0.17	1.46	1.02	1.02	0.75

[1] A dried uncooked product made by Swift and Company.

[2] Average data obtained on two normal dogs.

The fraction of absorbed nitrogen retained in the body of the animal (BV) is defined strictly by the following equation.

$$NB = (BV)\ (AN) - EN \qquad (5)$$

where EN is the fraction of nitrogen excreted which can be attributed to body nitrogen ("endogenous"). For any given value for NB, equations (1) and (5) can be solved simultaneosuly to give:

$$EN = NE_o - AN\ (K - BV) \qquad (6)$$

Equation (6) proves that if K in equation (1) is greater than BV, then EN becomes less than NE_o. In this way egg white can conserve body nitrogen. Similarly conservation of body nitrogen by egg white in rats has been reported by Willman, Swanson, Stewart, Stevenson, and Brush ('45). Fractions of egg white are being prepared to try to isolate the amino acids or other substances which produce this interesting effect.

Correlations between values of K and the excretion of nitrogen in the urine are illustrated by the data in figure 2. Daily excretion of urine nitrogen in gm./day/sq.M. are plotted in this figure, first when the animals were on a protein-free diet (open circles) followed by nitrogen feeding (solid circles) and then back on the protein-free diet (open circles). Curve I was obtained on a normal dog which received 1.84 gm./day/sq.M. of nitrogen in the casein hydrolysate [2] during an 8-day feeding period. K was less than unity for this hydrolysate, the excretion of nitrogen in the urine increasing, therefore, during the nitrogen feeding period above that found for the protein-free period. Curve II was obtained on a partially depleted dog receiving 1.44 gm./day/sq.M. of egg white nitrogen where the K value was very close to unity. When K is unity the excretion of urine nitrogen is independent of the quantity of food nitrogen fed. Curve III represents data obtained on a hypoproteinemic dog receiving 1.52 gm./day/sq.M. of egg white nitrogen. The K value being greater than unity, the urine nitrogen excreted during the nitrogen feeding period is less than during the protein-free feeding period.

The relationships expressed by equation (1) and the others suggests that regeneration of body proteins would be most marked when the animal is in positive nitrogen balance. The data in figure 3 support this suggestion. Here plasma protein regeneration areas ($\triangle$ plasma protein times day) are plotted against positive nitrogen balances produced. Regeneration areas,[6] described by Seeley ('45) and explained under methods in this paper are used to measure the effect of dietary pro-

[6] Some of these data were derived from Seeley ('45).

teins on regeneration of plasma proteins in the depleted animal. The circles with the crosses in figure 3 illustrate data obtained by feeding bovine serum protein, the lowest point being obtained with 1.6 gm. of nitrogen/day/sq.M. of body surface, the second lowest with 3.4 gm. and the two higher points with 6.6 gm./day/sq.M. The circles with horizontal lines, rectangles, square, and open circles illustrate data obtained by feeding 6.6 gm. of nitrogen/day/sq.M.

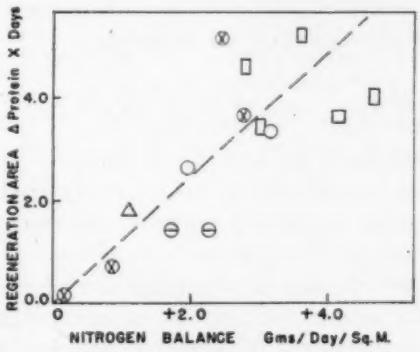

Fig. 3 Regeneration areas (△ protein times days) are plotted against positive nitrogen balances in gm./day/sq.M. The data was obtained on depleted dogs orally fed bovine serum protein ⊗, casein ○, casein hydrolysate ▯, hydrolysate B □ and hydrolysate C ⊖, respectively. The △ represents data obtained on a depleted dog fed hydrolysate B intravenously.

of commercial hydrolysate C,[4] casein hydrolysate,[2] commercial hydrolysate B,[3] and casein, respectively. The triangle describes one experiment when hydrolysate B was given intravenously at the rate of 8.0 gm./day/sq.M. These data prove that under the experimental conditions used, regeneration of plasma proteins increases regularly from practically zero near nitrogen equilibrium to high values when the dogs are kept well on the positive side of nitrogen balance. The amount of regeneration is a function of the nitrogen balance produced, and therefore of K and K′. These results support the conclusion of Melnick, Cowgill and Burack

('36), that there is a correlation between the nitrogen balance produced and the amount of plasma protein regeneration. The kind of plasma protein regenerated, however, will depend upon the state of depletion of the animal and the pattern of amino acids fed (Seeley, '45).

SUMMARY

The relationships between nitrogen balance and absorbed nitrogen and ingested nitrogen in normal and hypoproteinemic dogs are described by the equations:

$$NB = K\ (AN) - NE_o$$
$$NB = K'\ (NI) - NE_o$$

where NB is nitrogen balance, AN is absorbed nitrogen, NI is ingested nitrogen, and NE_o is the excretion of nitrogen on a protein-free diet. These are linear relationships, within experimental error, in the regions of negative and low positive nitrogen balance. K and K′ are functions of the retention of nitrogen by the animal and may be called biological values or nitrogen balance indexes of absorbed and ingested nitrogen respectively.

The values for K, K′ and "net protein" value increase, while those for absorbed nitrogen at equilibrium and NE_o decrease from normal in the hypoproteinemic dog.

These constants are functions of, but not necessarily the fractions of, nitrogen retained in the body of the animal since values greater than unity have been obtained while feeding egg white to hypoproteinemic dogs. Continued feeding of egg white results in a decrease in the excretion of nitrogen on a protein-free diet (NE_o). The data can be interpreted to mean that egg white spares body nitrogen.

Regeneration of plasma protein increases in magnitude as the nitrogen balance increases on the positive side. Thus regeneration of tissue proteins in hypoproteinemic dogs as reflected in the increase of plasma proteins is a function of the nitrogen balance produced and therefore of K or K′.

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THE EFFECT OF CHANGES IN DIET ON THE VOLUME AND COMPOSITION OF RAT MILK[1]

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FOUR FIGURES

(Received for publication September 10, 1945)

The availability of a method to obtain milk from rats (Cox and Mueller, '37) permits a new approach to studies on lactation. The present report deals with the variations in the composition of milk from rats on a standard diet, and the effect on yield and composition induced by variations in the protein, yeast and calcium and phosphorus levels. The composition of cow's milk must be related to the fact that the cow is a herbivore and that the major constituents of her diet are hay and grass. Changes in the diet of the cow are effected by change in the type of feed which may itself introduce changes other than the single constituent under observation. Thus, for example, two hays of different calcium content may be used in obtaining different calcium levels, and at the same time the proteins of the two hays may be of different nutritional value. Such difficulties are avoided when the rat is used as the experimental animal.

EXPERIMENTAL PROCEDURE AND RESULTS

Stock rats. Thirty mature stock rats were milked once, 18–20 days after parturition and the samples were each ana-

[1] Presented at the Memphis meeting of the American Society of Biological Chemists, April 21–24, 1937.

lyzed for fat and total protein. Eighteen additional rats were similarly milked and calcium, phosphorus and magnesium determined on each sample. The data are summarized in table 1.

The range of values for each constituent seems quite wide — but it is of the order commonly observed in surveys of the composition of cow's milk (Tocher, '25). Milk from individual cows varies widely in composition and even the pooled milk from twenty-four cows may vary as much as 1.0% in butter fat (H.M.S.O., '35). The coefficients of variation given (table 1) indicate that the protein, ash and magnesium content of rats' milk are subject to the least fluctuation and, as might be

TABLE 1

Variations in the composition of milk from individual stock rats.

	NO. RATS	MEAN	RANGE	STANDARD DEVIATION	STANDARD [1] ERROR	COEFFICIENT OF VARIATION
Volume (ml./rat)	30	3.2	1.0 – 5.9	1.4	± 0.26	43.7
Fat %	30	13.3	7.1 –19.6	3.25	± 0.59	24.4
Protein % (N × 6.25)	30	10.4	8.4 –14.8	1.41	± 0.26	13.5
Ash %	18	1.43	1.15 – 1.83	0.165	± 0.040	11.5
Calcium %	18	0.325	0.242– 0.447	0.0585	± 0.014	18.0
Phosphorus %	18	0.232	0.175– 0.311	0.0395	± 0.0096	17.0
Magnesium %	18	0.025	0.021– 0.032	0.0028	± 0.00068	11.2

[1] $\frac{\sigma}{\sqrt{N}}$ used for first 3 rows; $\frac{\sigma}{\sqrt{N-1}}$ for last four.

expected, the widest variation was in the amount of milk obtained. There is some indication that the percentage protein varies with the volume of milk. The correlation coefficient was — 0.52, which is 5.8 times its probable error. This is similar to findings on cow's milk (Tocher, '25).

In the course of work to be described later, analyses for three milk constituents for two successive milkings from the same rat were made (18th and 20th day of lactation). An opportunity was thus available for determining whether the day to day variation in the composition of the milk from a single rat was equal to the differences observed between single

samples from individual rats. The fact that the rats so studied were on various diets does not detract from the validity of the differences between two values on the same individual. The standard deviations for the per cent fat, protein, and ash in rat milk (from table 1) are recorded in table 2 in order that they may be compared with the standard deviation in fat, protein and ash content of pairs of determinations on single rats. To calculate this value, the usual formula $\sqrt{\frac{\Sigma d^2}{N}}$ was applied to the plus or minus variation of the second from the first determination. In this way the first of each pair of observations served as the base.

TABLE 2

Variations in the composition of milk obtained on successive milkings.

CONSTITUENT	RATS MILKED ONLY ONCE		RATS MILKED ON 18TH AND 20TH DAYS	
	No. obs.	Standard deviation	No. obs.	Standard deviation [1]
Fat	30	3.25	28	2.70
Protein	30	1.41	35	1.46
Ash	18	0.165	29	0.199

[1] Calculated by algebraically subtracting the second determination of each pair of observations from the first and determining σ.

The data indicate that the day to day variation in the composition of milk is as great as the variation when single samples are obtained. It is impossible to obtain an entire 24-hour milk sample from rats and thus a strict comparison with cow's milk cannot be made.

Effect of dietary protein. Ten young mother rats were placed at parturition on each of six diets which differed only in protein (casein) content. Levels of 5, 10, 20, 30 and 50% casein were incorporated in diet no. 1 of Cox and Imboden ('36). The mother rats were milked on the eighteenth and twentieth days of lactation and as many analyses (for protein, fat and ash) run as was consistent with the volume of milk obtained.

The average total protein content (N. $\times$ 6.25) of the milk from the five groups was 9.8^{5}, 9.6^{13}, 9.7^{15}, 10.0^{12}, and 10.7^{16}, respectively. (The prime figures indicate the number of determinations.) While there seems to be a small increase in the protein content with increasing intake, the differences are small and not statistically significant. This is in agreement with the general findings of Perkins ('32) on cows and others (Meigs, '22). Adair ('25) observed that human mothers on a high protein diet produced more milk than did mothers on a high fat or high carbohydrate diet. No analyses of the milk were reported.

Other constituents of the milk were somewhat modified by the protein intake. The per cent ash, per cent fat, the average volume of milk and the number and weight of young successfully raised to 21 days of age are given in figure 1. With the exception of the difference in the per cent milk fat from rats on the 20- and 30%-level, the differences in this constituent are statistically significant, the significance ratio $\frac{M_1 - M_2}{\sqrt{P.E._{M_1}^2 + P.E._{M_2}^2}}$ falling between 3.9 and 4.9. The variations in ash content follow the trend of fat, but the differences for the former are not significant.

As indicated in the curve, the volume of milk at low protein levels is greatly reduced, and this necessarily limits the growth and weight of young. It is interesting, however, that the average number of young raised by mothers on low protein diets was only slightly less than the number raised on diets higher in protein, although their weight was markedly reduced.

Calcium and phosphorus. One of the authors (Cox) and Imboden ('36) studied the effect of different levels and ratios of calcium and phosphorus on reproductive success. These studies were continued through the entire reproductive life of the rats (ten pregnancy cycles) and cannot be strictly compared with the observations below because a rat may raise one or more litters on a diet inadequate in calcium and phosphorus with fair success, so that its inadequacy may be fully

demonstrated only in later life. It was believed of interest, however, to determine the degree to which the composition of milk could be influenced by the more severe rations employed in the earlier work. It was hoped that further information on the tolerance for high phosphorus rations at low calcium intakes might be obtained. The rations employed are given in table 3.

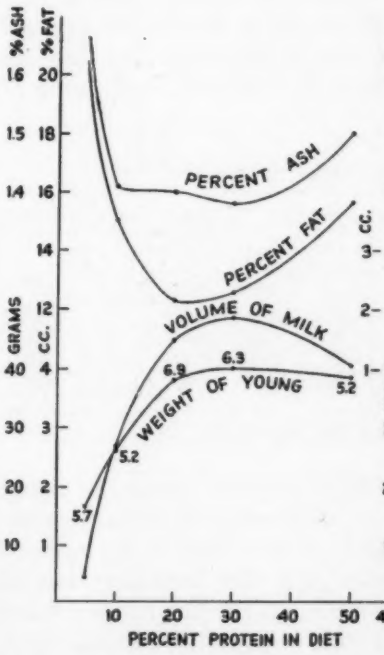

Figure 1

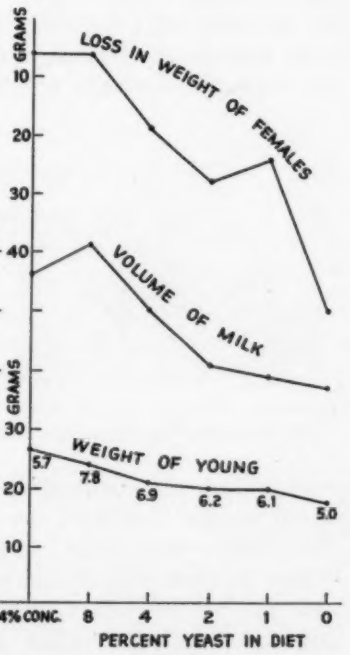

Figure 4

Fig. 1 The effect of the protein level of the maternal diet on the volume of milk obtained under standard conditions, the weight and number of young raised to 21 days, the ash and percentage fat of the milk. The values on the curve indicate the average number of young raised to 21 days. Ten rats were used on each diet.

Fig. 4 The effect of reducing the yeast intake of lactating mother rats as measured by their loss in weight, volume of milk and weight and number of young. Yeast or yeast concentrate was the only source of B-vitamins in the diet. The values on the curve indicate the average number of young raised by the ten rats on each diet.

The experimental procedure was exactly the same as that employed in the studies just described, save that all diets contained 20% protein. The data are presented graphically in figures 2 and 3 and confirm the earlier finding that diets with high Ca/P ratios are not as well tolerated as diets equally unbalanced as regards phosphorus. On the high calcium diets (right hand portion of the curves) the mothers lost a great deal of weight in an effort to raise their young — even though the offspring were definitely subnormal in weight and appearance. On the other hand, the high phosphorus diets did not deleteriously affect the weight of the mothers, and young of

TABLE 3

Levels and ratios of calcium and phosphorus employed.

DIET NO.	CALCIUM	PHOSPHORUS	CA/P RATIO	"SUCCESS RATING"[1]	DIET NO.	CALCIUM	PHOSPHORUS	CA/P RATIO	"SUCCESS RATING"[1]
	%	%				%	%		
1	0.245	0.245	1.0	76.0	6	0.490	0.245	2.0	90.4
2	0.245	0.490	0.5	79.9	11	0.735	0.245	3.0	72.8
3	0.245	0.735	0.33	93.6	16	1.225	0.245	5.0	13.2
4	0.245	1.225	0.2	83.5	21	2.450	0.245	10.0	0.5
5	0.245	2.450	0.1	10.3	Stock	0.45	0.47	1.1	89.0

[1] On 10 reproductive cycles (see table 2, Cox and Imboden, '36).

good average size resulted. The difference in number of young raised on the high phosphorus diets may be fortuitous because more young were raised on diets with a P/Ca ratio of 10 than on diets with P/Ca ratios of 3. The largest volume of milk was obtained at a Ca/P ratio of 2.0, but ratios higher than this resulted in rapid decline in milk production. This was not the case with diets unbalanced in terms of phosphorus.

The composition of the milk was influenced only slightly, as indicated in figure 3. High phosphorus diets had practically no effect on the Ca, P, total ash or Ca/P ratio of the milk. With increase in dietary Ca/P ratio, however, there was progressive decrease in total ash, calcium and phosphorus. In this portion of the curve the Ca/P ratio of the milk increased with the Ca/P ratio of the diet, simultaneously with

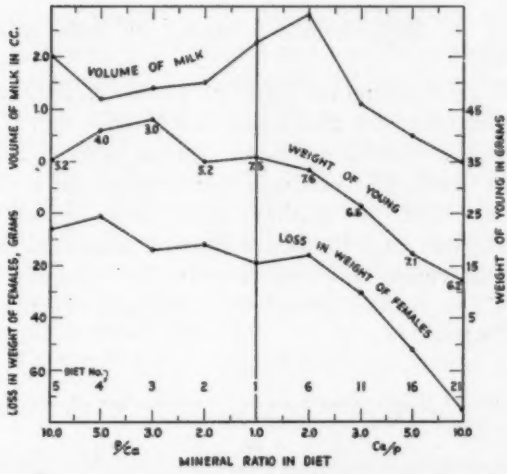
VOLUME OF MILK
WEIGHT OF YOUNG
LOSS IN WEIGHT OF FEMALES
VOLUME OF MILK IN CC.
LOSS IN WEIGHT OF FEMALES, GRAMS
WEIGHT OF YOUNG IN GRAMS
DIET No.
5 4 3 2 1 6 11 16 21
10.0 5.0 3.0 2.0 1.0 2.0 3.0 5.0 10.0
P/Ca
Ca/P
MINERAL RATIO IN DIET

Fig. 2 The effect of various calcium and phosphorus levels in the maternal diet on the volume milk, the weight and number of young raised to 21 days of age, and the change in weight of the mother rats during lactation. High phosphorus diets are on the left, and high calcium diets on the right of the center line. At a Ca/P ratio of 1.0, the diet contained 0.245% calcium and 0.245% phosphorus. The values on the curve indicate the average number of young raised by the ten rats on each diet.

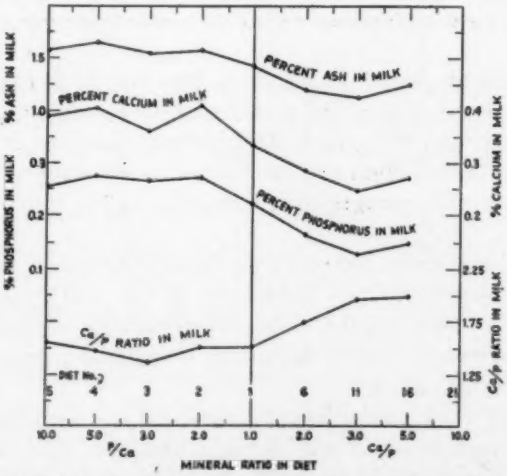
PERCENT ASH IN MILK
PERCENT CALCIUM IN MILK
PERCENT PHOSPHORUS IN MILK
Ca/P RATIO IN MILK
% ASH IN MILK
% PHOSPHORUS IN MILK
% CALCIUM IN MILK
Ca/P RATIO IN MILK
DIET No.
5 4 3 2 1 6 11 16 21
10.0 5.0 3.0 2.0 1.0 2.0 3.0 5.0 10.0
P/Ca
Ca/P
MINERAL RATIO IN DIET

Fig. 3 The effect of changes in calcium and phosphorus levels of the maternal diet on the inorganic constituents of the milk.

a decrease in the absolute amounts of both calcium and phosphorus.

Yeast. To determine the effect of yeast, ten rats at parturition were placed on the basal diet (no. 1) save that the protein level was adjusted to 10% casein. Levels of 8, 4, 2, 1 and 0% yeast were used. The controls received 4% of a B-complex concentrate. Half the mothers were kept on wire screens during lactation, and the other half on shavings, but as no essential difference was observed in the behavior of these subgroups, the results have been averaged for presentation (fig. 4 and table 4).

TABLE 4

Effect of decreasing yeast on the composition of milk.[1]

DIET NO.	YEAST	FAT	PROTEIN	ASH
		%	%	%
60	4% Conc.	16.40(19)	9.71(20)	1.40(18)
61	8	15.16 (8)	8.83 (8)	1.24 (8)
62	4	15.46(14)	8.91(14)	1.10(13)
63	2	15.49 (7)	9.10 (8)	1.26 (7)
64	1	15.49(10)	9.74(10)	1.10 (8)
65	0	17.63 (8)	10.15 (8)	1.28 (6)

[1] The figures within parentheses indicate the number of determinations.

The limiting factor in all diets was the 10% casein level, and this accounts for the small size of young on 4% yeast concentrate, and 8% yeast. Differences from the findings on these two control diets are attributed to the level of yeast, although there was progressive reduction in the intake of yeast protein.

It is evident from figure 4 that decreasing amounts of yeast (the sole source of the B-factors in the diet) constitute a progressive drain on the maternal organism, as indicated by her loss in weight during lactation, a reduced volume of milk, and the size of young.

The fact that young could be raised on no yeast intake, during a period when the B-requirement is increased, is a point of considerable interest. It is generally believed that

vitamin B is not stored in the tissues so that the partial success in lactation on a B-deficient diet may be an expression of the time necessary for a B-deficiency to develop, or is related to the intestinal bacterial synthesis of B as recently reported by Najjar and Holt ('43). It should also be noted that the maximum effect of lack of yeast was in the loss in weight of the mothers, and in reduced milk volume rather than in any large weight difference in the offspring. The weight of young for 4, 2 and 1% yeast was the same, but this was accomplished at the expense of the weight of the mother.

Table 4 gives the percentages of fat, protein and ash in the milk. No significant change in any component was noted.

DISCUSSION

Scientific feeding for milk production is based on such a volume of work that it is not surprising that observations with rats do not disclose hitherto unrecognized facts. This applies particularly to the study of protein level, but it is of interest to know that the dietary factors affecting milk secretion in omnivora are similar to those which operate in herbivora. Thus, increase in protein intake does not materially change the per cent of milk protein, but does definitely increase milk volume. This is apparently true in cows, rats and humans. In our experiments increase in milk volume resulted in larger young but this could not be demonstrated in Adair's ('25) human observations (probably because of complemental feedings).

In spite of wide variations in mineral intake, the Ca/P ratio of rats' milk is maintained fairly constant between 1.4 and 1.7 although the total range was from 1.4 to 2.0. The chief effect of high calcium low phosphorus diets is not only to increase the Ca/P ratio of the milk but more significantly to decrease the total amount of calcium secreted. Thus, on diets numbered 1, 2, 3, 4, 5 and 6, a total of 5–10 mg. of calcium was secreted (under the technique described), while on diets 11 and 16, only 2.6 and 1.3 mg., respectively, was secreted. Thus, when

rachitogenic diets are fed, the young suffer most acutely from a calcium deficiency and therefore do not grow.

Corresponding to the low absolute amount of calcium secreted, the per cent of calcium in the milk on these high calcium rations was low (fig. 3). The number of determinations on each diet was not large (5–8) and individual variation in rats resulted in a large standard deviation. The differences between adjacent points (as between diets 1 and 6, and 6 and 11) have no statistical significance (significance ratios were 1.6 and 2.0, respectively) but the significance ratio for diets 1 and 11 was 3.1. The trend is sufficiently distinct to indicate the detrimental effect of high calcium diets on the calcium content of the milk. The food intake of lactating rats is not easily measurable but qualitative observations indicated that with the exception of diet no. 21 (table 3) the rats on the mineralized rations and on those containing different levels of yeast, consumed approximately the usual amount of food.

SUMMARY AND CONCLUSIONS

Milk from individual stock rats has been analyzed for protein, fat, ash, calcium, phosphorus and magnesium.

The effect of changes in the diet of lactating rats has been studied by observing the change in composition of the milk, the number and size of young raised, and the effect of the diet on the mother rat. It is concluded that:

1. Increase in the protein intake results in increased milk yield, but no significant change in the per cent protein in the milk.

2. High calcium diets result in decrease in both milk volume and the percentage of calcium and phosphorus in the milk, with consequent decrease in the total amount of calcium and phosphorus secreted. High phosphorus diets had no demonstrable effect on these milk constituents.

3. Decrease in the intake of yeast decreases the volume of milk, the size of nursing young, and causes severe weight loss in the lactating mothers, without changing significantly the composition of the milk.

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